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REMOVAL OF POTENTIAL INHIBITORS FROM HEMICELLULOSE HYDROLYSATE BY MEMBRANE FILTRATION

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Ce mémoire intitulé:

REMOVAL OF POTENTIAL INHIBITORS FROM HEMICELLULOSE HYDROLYSATE BY MEMBRANE FILTRATION

présenté par : CHEGINI Salomeh

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DEDICATION

To my beloved dad and mom: Iraj and Derakhshandeh

To my Love: Byandor

To my darling brother and sister: Mehdi and Sormeh

For their endless love and support

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I owe sincere thankfulness to my research supervisors, Professor Jean Paris, Professor Mario Jolicoeur and Dr. Mariya Marinova for their understanding and encouragement. I am sure that this research would not have been possible without their supports and patience.

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RÉSUMÉ

Au cours des dernières années, avec l'augmentation des préoccupations environnementales et des sources limitées de combustibles fossiles, la production des biocarburants a attiré une attention croissante. Le bio-butanol, en raison de ses nombreux avantages par rapport aux autres biocarburants, peut être considéré comme une alternative appropriée. Le bio- butanol peut être produit à partir des sucres pentoses et hexoses par la fermentation acétone-butanol-éthanol (ABE), un procédé anaérobie qui utilise généralement des bactéries à partir des souches de Clostridium, pour produire de l'acétone, de butanol et d'éthanol dans un rapport de 3-6-1 respectivement. Un inconvénient de ce procédé est le faible rendement et l'une des raisons est la présence d'une large gamme de composés tels que les furanes, les acides organiques faibles et des composés phénoliques qui sont toxiques et qui empêchent la fermentation efficace. Surmonter l'impact de ces inhibiteurs est l'un des principaux défis pour la production de butanol. À cet effet, l'élimination presque complète de ces groupes de produits chimiques est une nécessité. La détoxification est une étape très critique.

L'objectif principal de ce travail a été d'étudier l'efficacité de la filtration par membrane pour éliminer les inhibiteurs potentiels contenus dans l'hydrolysat hémicellulosique et améliorer le pouvoir fermentescible des sucres pour la production du bio -butanol.

Un mélange de pré-hydrolysat de l'érable-tremble a été utilisé comme solution initiale dans toutes les expériences. Cinq membranes couvrant une large gamme de seuils de coupures (MWCO) (entre 100 et 10000 daltons) ont été testées. Trois membranes sont de type nanofiltration (NF90, NF270 et XN45) et deux de type ultrafiltration (UA60 et UE10.

Dans ce travail, deux scénarios ont été étudiés: Le premier a consisté en l'évaluation des performances des étapes de concentration et détoxification après l'hydrolyse acide du pré - hydrolysat et le deuxième a été consacré à la concentration – détoxification avant l'étape de l'hydrolyse acide. Les variations de la concentration des sucres réducteurs et des composés inhibiteurs ont été déterminées dans les deux scénarios et l'efficacité des membranes testées a été comparée.

Chacun des deux scénarios a comporté deux étapes. La première a été consacrée à la sélection de la membrane la plus efficace (meilleur taux de rétention des sucres et meilleur taux de passage des inhibiteurs dans le perméat). Les essais ont été réalisés en boucle fermé (le concentré et le

perméat sont retournés dans la cuve d'alimentation) pour évaluer seulement les performances de séparation des membranes. La deuxième étape a été consacrée à la réalisation des essais de concentration et de détoxification avec la membrane sélectionnée lors de l'étape précédente.

Dans les deux scénarios, les résultats des essais de sélection de la membrane la plus efficace ont monté que les meilleurs taux de conservation des sucres et les taux d'élimination des inhibiteurs les plus élevés ont été obtenus avec la membrane XN45. Aussi, la membrane XN45 a été sélectionnée pour les étapes subséquentes prévues dans les deux scénarios. Ces derniers ont consistés en une concentration – détoxification du pré-hydrolysat et de l'hydrolysat acide. Afin d'augmenter les taux de détoxification, la procédure de filtration a été complétée par une étape de dia-filtration.

Dans le cas de l'hydrolysat acide, l'expérience a consistée en l'extraction de 0.85L de perméat à partir de 1.7L de solution initiale (soit un facteur de concentration de 2 fois). Une fois le taux de concentration atteint, l'expérience a été complétée par une dia-filtration avec un volume d'eau déminéralisée de 2 fois le volume du concentré. Une expérience similaire a été réalisée avec le pré-hydrolysat (extraction de 1L de perméat à partir de 2L de pré-hydrolysat et dia-filtration avec 1L d'eau déminéralisée). La variation de la concentration de sucres réducteurs et les composés inhibiteurs a été déterminée dans les deux scénarios et l'efficacité des membranes testées a été comparée. Dans ces expériences, afin de retenir davantage de sucres et de retirer plusieurs inhibiteurs, dans les deux scénarios, la nano - membrane XN45 présentait de meilleures performances. Lors de l'étape suivante, pour la rétention des inhibiteurs et l'obtention de la concentration la plus élevée de sucres, le processus de concentration suivie d'une dia - filtration a été appliqué.

Cette étude a démontrée que la dia - nanofiltration est un procédé efficace qui permet l'élimination de l'acide acétique, le furfural et HMF, cependant la séparation membranaire, même en combinaison avec la dia - filtration ne permet pas de séparer les composés phénoliques. En outre, les résultats de notre étude ont démontré que le premier scénario

(concentration après hydrolyse) est une approche prometteuse afin d'obtenir une alimentation appropriée pour l'étape de fermentation (concentration en sucre plus élevée et moins d'inhibiteurs). La dernière étape consistait à étudier la fermentabilité de l'hydrolysat après la détoxification et mesurer l'efficacité des procédés de désintoxication sur la croissance microbienne et la bioconversion des sucres générés durant l'hydrolyse en bio-butanol. Lors de

cette étape, des voies d'optimisation et de validation de l'efficacité des procédés de détoxification de l'hyrolysat par voie membranaire ont été explorées.

ABSTRACT

In recent years, with rising environmental concerns and limited sources of fossil fuel, bio-fuel production has attracted growing attention. Bio-butanol is a bio-fuel that is an alternative to fossil fuels and can be produced by fermentation of pentose and hexose sugars through acetone-butanol-ethanol (ABE) anaerobic fermentation process, which uses bacteria from the clostridium strains to produce acetone, butanol and ethanol in a ratio of 3-6-1, respectively. A main drawback to this process is its relatively low yield, which results in part from the presence of a broad range of compounds such as furans, weak organic acids and phenolic components. These components are toxic and prevent efficient fermentation. Overcoming the impact of inhibitors is one of the main challenges for bio-butanol production, and since near complete removal of these groups of chemicals is a necessity detoxification is a critical step. The main objective of this project is to study the efficiency of membrane filtration for removing the potential inhibitors from hemicellulosic hydrolysates in order to improve its fermentability for bio-butanol production.

In this work, a pre-hydrolysate of maple-aspen blend was used as an initial solution. A number of experiments were performed using five membranes, with broad range of molecular weight cut-off from 100 to 10,000 Daltons, including nano-filtration (NF) membranes and ultra-filtration (UF) membranes. Two scenarios also were conducted: the first one was concentration after hydrolysis and the second one was concentration before hydrolysis. The change in the concentrations of reducing sugars and inhibitory compounds were determined in both scenarios, and the efficiency of tested membranes was compared. In these experiments, in terms of retaining more sugar and removing more inhibitors, the nano-membrane XN45 exhibited better performance in both scenarios, and was selected as a suitable membrane in the subsequent steps.

Moreover, in order to get higher sugar concentration and remove more inhibitors, the concentration process followed by dia-filtration step(s) was applied.

In this study, dia-nanofiltration showed to be an efficient process to remove acetic acid, furfural and HMF. However, the performance of membrane separation, even in combination with dia-filtration process, was less effective in terms of phenolic elimination.

Furthermore, the results from our study demonstrated that the first scenario (concentration after hydrolysis) was a more promising approach to get a suitable feed for the fermentation step

(higher sugar concentration with lower inhibitor contents). In the final step, to investigate the effects of the applied detoxification process on the microbial growth, the detoxified samples were subjected to fermentation process.

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LIST OF SYMBOLS AND ABBREVIATIONS

ABE	Acetone-Butanol-Ethanol
Ai	Initial feed
ATCC	American type culture collection
C feed	Solute concentration in feed (g/L)
C permeate	Solute concentration in permeate (g/L)
HMF	Hydroxymethylfurfural
MWCO	Molecular weight cut-off
P0	Permeate sample at beginning of filtration
P30	Permeate sample after 30 minutes
P60	Permeate sample after 60 minutes
P90	Permeate sample after 90 minutes
RCM	Reinforced Clostridial Medium
SM	Synthetic medium
(δ/m)	Siemens per meter: unit of conductivity in SI

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Chapter 1 **INTRODUCTION**

1.1 Background

The pulp and paper industry has always been considered as an important part of the Canadian economy. However, in the recent decades, due to the low price of paper, rising energy costs and a decrease in the demand for traditional P&P products, this industry has had to face some economic challenges (Montastruc et al., 2011). One strategy to overcome this situation and make it an advantage, is converting the exiting chemical pulp mills into the Integrated Forest Bio-refineries (IFBR), which by using renewable feedstock and applying innovative technologies can produce higher value-added products (Florbela Carvalheiro et al., 2008; Colodette et al.; Longue Júnior et al., 2013; Montastruc et al., 2011; Van Heiningen, 2006).

The most used process to produce pulp from woody biomass is the Kraft pulping process (Vena et al., 2010). The objective of the pulping process is to eliminate lignin and keep the polysaccharides, specifically the cellulose fibers to produce pulp and paper (Fengel et al., 1983). In a typical Kraft pulping process (Figure 1-1), the wood chips are firstly subjected to white liquor (a solution of NaOH and Na_2S) for cooking. During the cooking step, several complex chemical reactions happen under high operational conditions and as a result, the major parts of lignin and hemicelluloses (almost 40-50% of the wood biomass) are dissolved and form black liquor. This black liquor, which contains degraded lignin, hemicelluloses and the spent cooking chemicals, is sent to the recovery boiler to be burned and generates steam and electricity (Vena et al., 2010). In the pulping process, although the wood chips lose majority of their lignin and around half of their total solid contents, they still can keep their physical construction. However, the structure is not strong enough and after performing several mechanical and chemical steps such as washing, bleaching and drying, they will degrade to the single fibers, which can be sold as market pulp (Yoon et al., 2011).

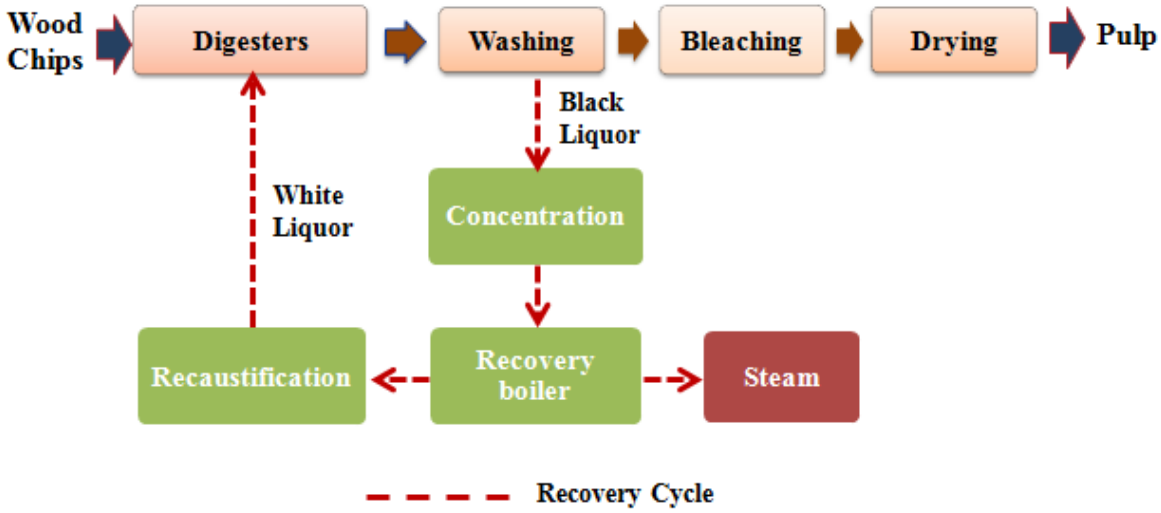


Figure 1-1: Simplified diagram of the conventional Kraft pulping process (Marinova et al., 2009)

Due to the low heating value of hemicelluloses (13.6 MJ/kg) compared to lignin (27.0 MJ/kg), the hemicelluloses underutilization in the existing pulping processes is evident, and burning of hemicellulose in the recovery boiler is not efficient (Longue Júnior et al., 2013; Van Heiningen, 2006; Vena et al., 2010). Moreover, hemicelluloses, as an inexpensive and abundant raw material, have potential for being suitable fermentation substrate to produce value added products such as bio-fuels. Therefore, the extraction of hemicelluloses, which would have been dissolved in the black liquor during pulping process, is an attractive alternative for pulp and paper mills, as they can increase their net revenue by producing bio-fuels, biopolymers, paper additives, and other chemicals in addition to their main products (Canilha et al., 2013; Saha, 2003).

The sustainable conversion of biomass into energy and other valuable products is defined as bio-refinery, a facility or facilities network, which integrates biomass transformation processes and equipment to generate power, bio-fuels, heat, and marketable chemicals from biomass (Cherubini, 2010; Stöcker, 2008). Figure 1-2 illustrates the biorefinery concept.

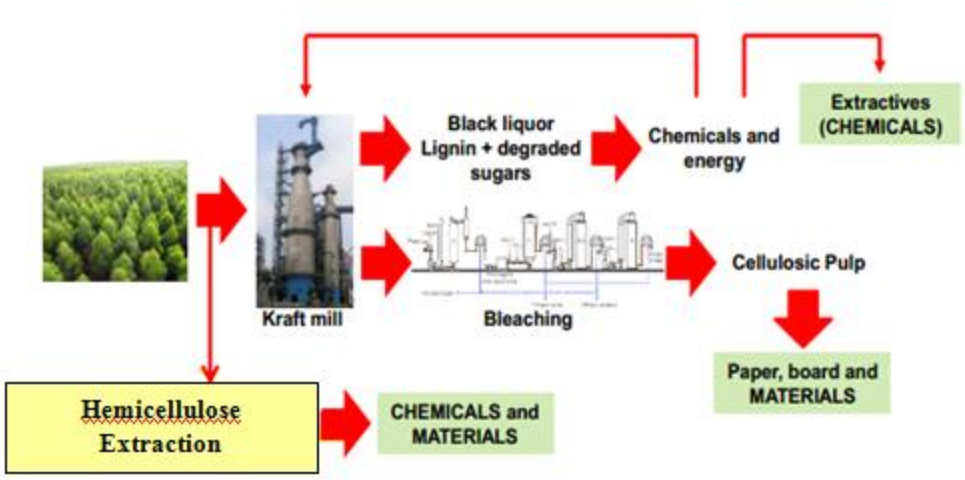


Figure 1-2: Bio-refinery concept (Longue Júnior et al., 2013)

A main problem associated with the bio-fuel production from hemicelluloses is the formation of toxic compounds, which inhibit microorganism metabolism and prevent efficient fermentation (Larsson et al., 1999). Thus, to overcome the inhibitory effects of these compounds, the use of a proper detoxification step prior to fermentation, plays an important role to improve the yield of bio-fuel production (Florbela Carvalheiro et al., 2008; Anuj Kumar Chandel et al., 2007).

Over the years, different detoxification methods such as physical (evaporation, membrane mediated detoxification), chemical (neutralization, calcium hydroxide over liming, activated charcoal treatment and ion exchange resins), and biological (enzymatic) have been developed. Each method has its own specificity to eliminate particular inhibitors from hemicelluloses hydrolysates. In this study, detoxification by membrane filtration was used to partially or completely removal of some potential toxic compounds from hemicellulosic hydrolysates and pre-hydrolysates. Furthermore, the growth of *Clostridium acetobutylicum*, a butanol-producing microorganism, on the concentrated-detoxified solutions was studied.

The current study has been done in collaboration with Centre National en Électrochimie et en Technologies Environnementales (CNETE) and FPInnovations. The pre-hydrolysate solution was provided by FPInnovations; the required experimental facilities were kindly supplied by CNETE and all the experimental work was performed there.

Chapter 2 LITERATURE REVIEW

2.1 Lignocellulosic biomass

Over the last few years, the environmental issues, national security and long-term economic concerns have attracted the research interests into biomass feedstock as an abundant source of fuels and chemicals, which are mainly produced from petroleum sources. Biomass most often refers to the plant or plant-derived materials like agriculture crops, trees and other biological substances that are derived from living, or recently living organisms (Alriksson et al., 2011; Canilha et al., 2013; Florbela Carvalheiro et al., 2008). The biomass is a renewable energy source, which includes carbohydrates. One of the most important biomass resources are lignocellulosic materials (Canilha et al., 2013) such as agricultural residues (hulls, straws, stalks, and stems), wastes of pulp and paper industry, coniferous and deciduous woods, municipal solid wastes, and herbal crops (Canilha et al., 2013; Saha, 2003). The lignocellulosic biomass contains cellulose, hemicelluloses and lignin (Figure 2-1); which build up to 90% of its dry matter along with smaller quantities of protein, peptin, ash and extractives (dissolvable materials including nitrogenous substance, non-structural sugars and waxes).

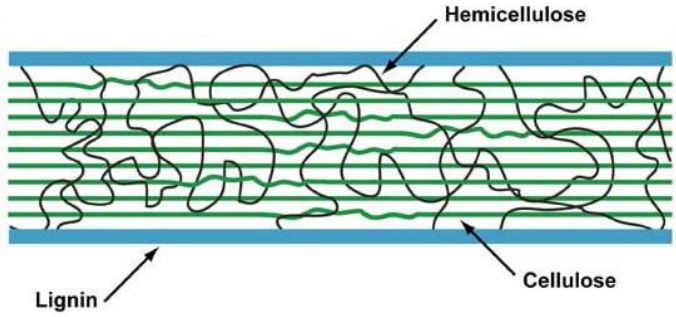


Figure 2-1 : The structure of lignocellulosic biomass
(Image: USDA Agricultural Research Service)

The composition of lignocellulosic biomass, based on some factors such as plant type and growing conditions, can differ in different plant species (Zhang et al., 2007, Saha, 2003). As an example, the composition and structure of hemicelluloses in hardwoods (e.g. aspen, oak and willow) and softwoods (e.g. pine and spruce) are different. The hardwood hemicelluloses contain

a higher portion of xylose (5-carbon sugar) than softwood hemicelluloses, which usually have higher glucose and mannose units (6-carbon sugars) (Kumar et al., 2009). Table 2-1 shows the composition of some lignocellulosic materials.

Table 2–1 : The structure of common lignocellulosic materials
(Kumar et al., 2009,Dehkhoda, 2008)

Lignocellulosic biomass	Cellulose (%wt.)	Hemicelluloses (%wt.)	Lignin (%wt.)
Hardwood	40-55	24-40	18-25
Softwood	45-50	25-35	25-35
Newspaper	40-55	25-40	18-30
Wheat straw	30	50	15
Switchgrass	45	31.4	12
Corn cobs	45	35	15
Grasses	25-40	35-50	10-30

The lignocelluloses materials account for almost 50% of the world biomass and has an estimated production of 10-50 billion t per year (Claassen et al., 1999). Generally, in the northern hemisphere, the dominant lignocellulosic materials are softwoods such as spruce and pine. In Canada, the commercial wood products are mainly made from softwoods; however, several value-added products are also delivered from hardwoods (Dehkhoda, 2008; Galbe et al., 2005).

2.1.1 Cellulose

Cellulose is the main fraction in the living plant cell walls. The most evident task of cellulose is to provide rigidity to the plant structure. Cellulose is a linear homo-polymer with D – glucose molecules that are bound by β (1-4) glycoside linkages (Figure 2-2) (Stöcker, 2008). Based on the source of cellulose, the length of macromolecules and degree of polymerization (DP) of cellulose can be varied. As an example, cotton presents an average DP of about 10000, while newsprint is investigated to have a DP of about 1000 (Roehr et al., 2001).

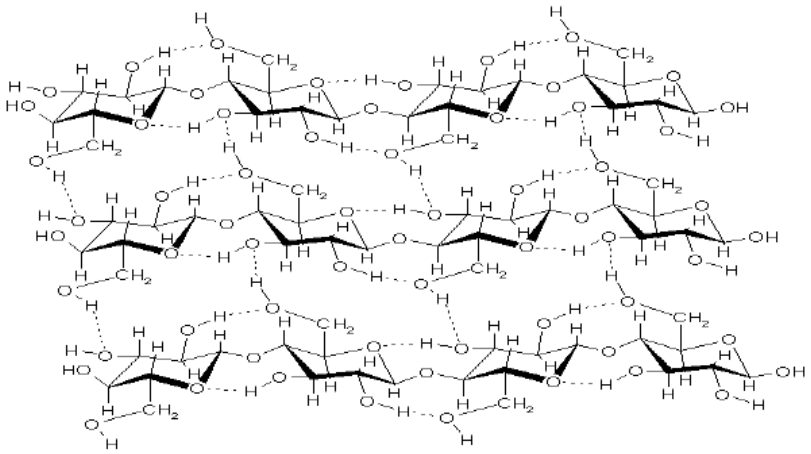


Figure 2-2 : Cellulose structure (Stöcker, 2008)

Based on its crystallinity degree, cellulose can be specified into crystalline and paracrystalline or amorphous cellulose. Cellulose can be converted to glucose by using enzymes (cellulases) or through chemical ways by means of acids such as sulphuric acid (Zheng et al., 2009). For degradation, the difficult parts in cellulosic structure are crystalline parts, while the easy regions for breaking down are amorphous parts (Dehkhoda, 2008; Roehr et al., 2001).

2.1.2 Hemicelluloses

Hemicelluloses are highly branched heterogeneous and amorphous polymers, which contain hexose sugars (monosaccharides carrying 6 carbon atoms e.g. glucose, mannose, galactose) and pentose sugars (monosaccharides carrying 5 carbon atoms e.g. xylose, arabinose). The hemicelluloses bound through hydrogen bonds to cellulose and covalently to lignin (Saha, 2003; Stöcker, 2008), and same as cellulose, can be hydrolyzed enzymatically (by hemicellulase) or chemically (e.g. by acids), and converted to its constituent monomers xylose, arabinose, galactose, glucose and / or mannose (Stöcker, 2008; Zheng et al., 2009). In comparison to cellulose, hemicelluloses have more amorphous areas and lower crystalline degrees that make them more sensitive to hydrolysis than to the hard cellulose structure (Dehkhoda, 2008).

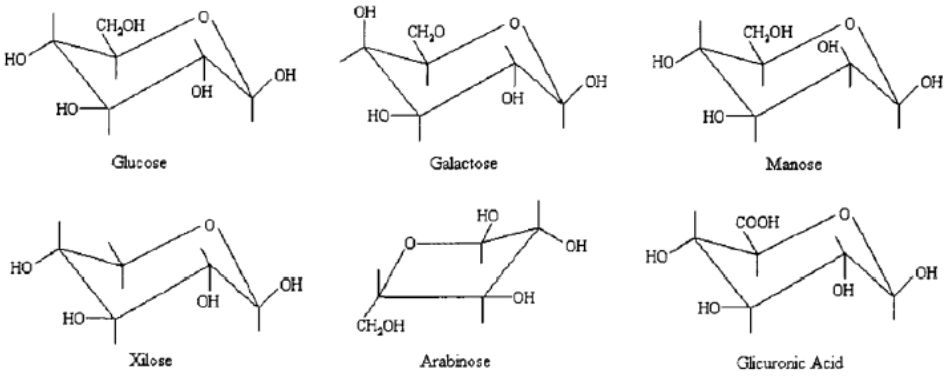


Figure 2-3: Monomers of hemicelluloses (Stöcker, 2008)

2.1.3 Lignin

Lignin is a highly complex three-dimensional polymer of several phenylpropanoid units, which are linked together by a variety of ether and carbon-carbon bounds (Martinez et al., 2009; Stöcker, 2008).

The lignin is formed by removing water from sugars through non-reversible reactions. This molecule with phenolic characteristic results from dehydration of three alcoholic monomers including: trans-sinapyl alcohol, p-coumaryl alcohol and trans-coniferyl alcohol, which bond together with ether bonds (Dehkhoda, 2008; Fardim et al., 2004).

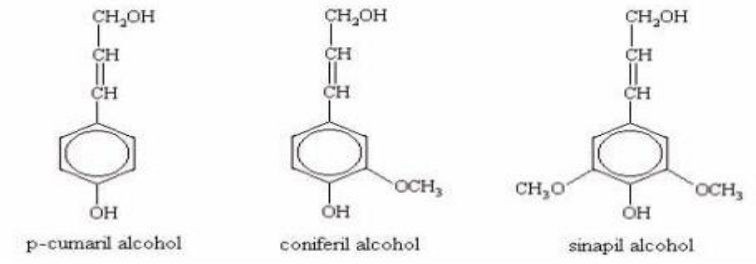


Figure 2-4: Monomers of lignin
(Zubieta et al., 2002, Palmqvist, 2000)

The lignin composition of different raw materials depends on their origin. As an example, the lignin content of softwoods (~30%) is higher than hardwoods (~20%) (Kumar et al., 2009).

Lignin, due to its complex molecular structure, is extremely recalcitrant towards biological and chemical degradation. The presence of lignin in lignocellulosic biomass, due to forming a protective wall, prevents the degradation of cell plants by bacteria and fungi (Kumar et al., 2009).

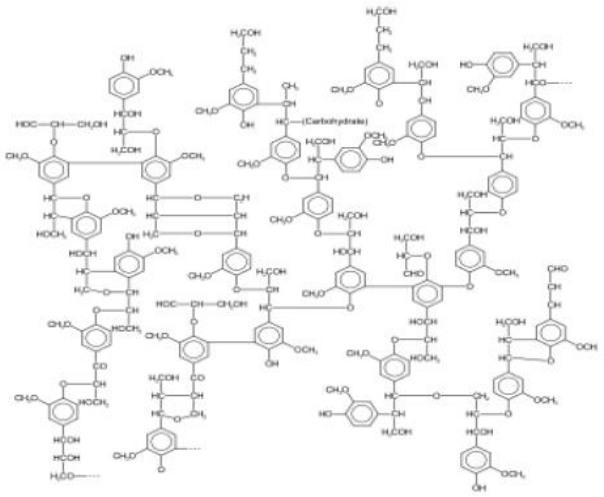


Figure 2-5: The structure of lignin (Kumar et al., 2009)

2.1.4 Extractives and ash

The extractives represent a low quantity (1-5%) of lignocellulosic biomass, which are non-cell wall materials and can be extracted by particular organic solvent. They include both lipophilic and hydrophilic constituents (Sjöström, 1993), and can be classified to phenolic extractives and wood resins (Dehkhoda, 2008; Sjöström, 1993). The resin can be observed in resin channel and pockets, whereas the phenolic extractives can be detected in the bark and inner section of woods. These compounds can be released during lignocellulosic pre-treatment, and despite their low amounts can have toxic effects on microorganisms (Dehkhoda, 2008).

2.2 Hemicelluloses applications

Hemicelluloses have a broad variety of applications (Figure 2-6). They can be converted to valuable products such as bio-fuel (e.g. ethanol and butanol), different biopolymers (e.g. polylactates and polyhydroxyalkanoates), and other value-added products such as xylitol and butanediol (Canilha et al., 2013).

Hemicelluloses can be employed as food additives, gelling agents, thickeners, emulsifiers, adsorbents, and adhesives (Canilha et al., 2013; Spiridon et al., 2005). In addition, hemicelluloses have been considered for their possible applications in medical industry such as anti-ulcer, wound protective and antitumor effects (Cipriani et al., 2006; Kardošová et al., 2002; Peng et al., 2012).

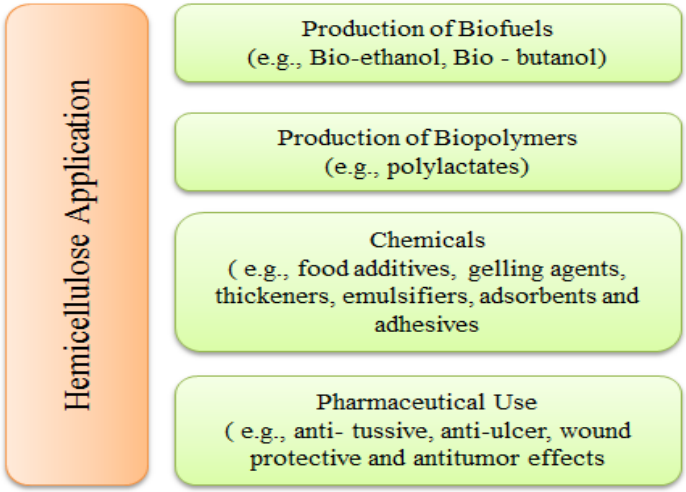


Figure 2-6 : Hemicelluloses applications

Recently, with the annual rise of global fossil fuels consumption, climate change and environmental concerns, limitation of crude oil resources, and the fluctuate price of crude oil, the bio-fuel production has attracted more attention (Anish et al., 2009; Smith, 2007). However, biomass-based fuels can represent just a part of the required fuels worldwide. Nevertheless, this fraction (e.g. in case of Germany, 25% is predicted for 2020) will significantly contribute to decrease greenhouse (Dürre, 2007; Paul et al., 2006). Moreover, for the market share of bio-fuels by 2020, a 10% minimum goal has been suggested (Cascone, 2008; Dürre, 2007; Liu et al., 2013).

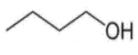
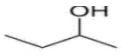
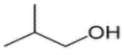
2.3 Butanol

Butanol or butyl alcohol, with a molecular formula of C_4H_9OH (MW 74.12), is a colorless and flammable alcohol with a banana-like odor. Butanol is an important chemical with a broad variety of applications. It can be applied as a solvent in drugs, vitamins, hormones, antibiotics,

cosmetics and household cleaners. Furthermore, butanol is traditionally used in polymer, paint and plastic industry as an industrial solvent.

Butanol has a structure with 4-carbons that the carbon atoms can form either a branched, chain or straight structure. Based on the carbon chain structure and OH location, different isomers with various properties can be formed. These isomers are n-Butanol, 2-Butanol, iso-Butanol and tert-Butanol (Dürre, 2007). Although the butanol isomers have different properties such as viscosity, boiling point and octane number, their basic applications are similar (industrial detergents, gasoline additives or solvents). The butanol isomers can be generated from fossil-based fuels by different techniques; however only n-butanol can be produced from biomass (Liu et al., 2013). The structure, applications and properties of butanol isomers are shown in Table 2-2.

Table 2–2 : Specifications of butanol isomers (Liu et al., 2013)

	n-Butanol	**2-Butanol**	**Iso-Butanol**	**Tert-Butanol**
Molecular structure				
Density (g/cm^3)	0.81	0.806	0.802	0.789
Boiling point(°C)	118	99.5	108	82.4
Melting point(°C)	-90	-115	-108	25-26
Octane number	78	32	94	89
Main applications	- Solvents for dye, paints, resins, etc. -Plasticizers, Cosmetics - Chemical intermediate for butyl esters or butyl ethers, etc. -Gasoline additive	-Solvent -Industrial cleaners -paint remover -Perfumes or in artificial flavors	-Solvent and additive for paint & Gasoline -Industrial cleaners -paint removers -Ink ingredient	-Solvent -Industrial cleaners -Paint removers -Gasoline additive for octane booster -Oxygenate intermediate for ETBE, MTBE, TBHP, etc.

2.3.1 Fuel properties and butanol advantages as a fuel

Butanol, in addition to its common applications such as being an extraction agent, chemical intermediate and industrial solvent, can be used as an alternative fuel for transportation vehicles (Cascone, 2008; Dürre, 2007; Ramey et al., 2004) . Butanol, as compared to ethanol, which is the conventional gasoline substitute, has more advantages. However, the main butanol properties

mainly depend on the type of isomer. Since n-butanol is the only butanol isomer, which can be produced from biomass through fermentation process, in this work, the considered fuel properties are those of n-butanol. The properties of n- butanol, ethanol and gasoline are compared in table 2-3 (Freeman et al., 1988; GREET, 2010; Liu et al., 2013; Speight, 2005).

Table 2–3 : Fuel properties

	Units	Gasoline	Ethanol	n- Butanol
Oxygen Content	%	Close to 0	36	22
Octane Number[1]	-	85-94	112.5-114	87
Reid Vapor Pressure	Bar	0.480-1.034	0.159	0.023
Higher heating value	MJ / Kg	46.5	29.8	37.3
Lower heating value	MJ / Kg	43.5	27	34.4

The standard vehicle engines are able to combust a fuel containing up to 15% ethanol (by volume) without any modifications of engines, while butanol with properties closer to those of gasoline can be mixed in any proportion. As an example, David Ramey travelled across the United States in a Buick with 100% butanol and without any engine modifications (Ramey et al., 2004).

Oxygen content is the second property of fuels that will be discussed. The concept is having more energy content in the fuel, which leads to decrease in carbon monoxide emissions and results in more complete combustion. Referring to the Table 2-3, the energy content of gasoline is almost zero, while ethanol and butanol contain 36% and 22% oxygen respectively, and can be employed as oxygenate agents and fuel additives (Liu et al., 2013; Szulczyk, 2010).

The next fuel property is the octane number, which is a measure of how much temperature and pressure is required to inflame the air-fuel blends. According to the Table 2-3, butanol has an octane level of 87, which is close to the octane number of gasoline. However, ethanol has a higher octane number that is an advantage. Thus, the petroleum distributors, in order to enhance the octane level, can blend a cheap and low-octane fuel with ethanol.

[1] average of The Octane Number is the Antiknock Index, which is the the Research Octane Number (RON) and Motor Octane Number (MON).

Another property of fuel is the Reid vapor pressure (a common measure of the fuel volatility). A fuel requires a minimum vapor pressure to be able to start a cold engine. Based on Table 2-3, ethanol has a higher Reid vapor pressure than butanol (Ramey et al., 2004). Therefore, butanol cannot vaporize simply, which may make it harder to start a cold engine. However, the easy fuel vaporization results in higher levels of pollution, specifically in hot summers, when the ultraviolet radiation of sun transforms the organic volatile components along with nitrogen oxides gases (NOX) into ground ozone pollution (EPA, 2011; Wu et al., 2007).

The energy content of the fuel is the other fuel specification that should be discussed. Fuel combustion generates the heat energy, which is converted to motion by car engines. Researchers use two heating value measurements: higher heating value (HHV) and lower heating value (LHV). The HHV includes the heat energy delivered containing the vaporization of water, while the LHV excludes the wasted energy on water vaporization. Usually, the lower heating value is used, because the energy of vaporized water cannot be utilized by car engines (Ramey et al., 2004). The lower energy content decreases the mileage[2]. Therefore, in terms of energy content, butanol (86%) has superiority to ethanol (65%). In addition, as compared to ethanol, the energy content of butanol is more similar to gasoline.

The final fuel property is fuel contamination and moisture. Ethanol is hydroscopic and liquid phase separation may occur in presence of water, while the blends of gasoline-butanol do not separate. Furthermore, ethanol as fuel cannot be preserved easily and the process of allocation, storage and transition is more difficult than that of gasoline.

To answer the question “why is butanol a better alternative?” we may say because its energy content is higher and it has a lower vapor pressure. Moreover, the blends of butanol-gasoline do not separate in the water presence, and butanol can be mixed with gasoline at any concentration without any engine modifications. In addition, the octane level of butanol is more comparable to gasoline and finally the butanol is less corrosive than ethanol (Liu et al., 2013; Ramey et al., 2004; Szulczyk, 2010).

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[2] Fuel Mileage : the ratio of the number of miles traveled to the number of gallons of fuel burned.

2.3.2 Butanol market

During recent years, the worldwide market of butanol has risen considerably, and mainly because of increasing demand in the Asia-pacific region.

As an example, in 2012 around 35% of the global butanol market belonged to China. However, the facilities of butanol production are mainly centralized in North America and Europe, which are the main butanol exporting areas in the world. In the near future, it is expected that the Middle East, due to the interests of their government in petrochemical and chemical companies, to be one of the main butanol global markets (Aster, 2012 ; Liu et al., 2013; Ramey et al., 2004; Szulczyk, 2010). The N-butanol consumption by different regions in 2012 has been shown in Figure 2-7.

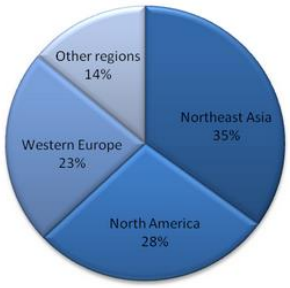

Figure 2-7 : N-butanol consumption by regions, 2011 (Aster, 2012)

The worldwide demand for n-butanol as a chemical is higher than 3.4 million tonnes per year, which is rising at over 4.4 percent each year. In addition, the market of butanol as a fuel blend stock has the possibility to grow to 122 million tonnes per year by 2020. Due to the unique potential of n-butanol to produce several value added products (e.g. butadiene, resins and 1-butene), it is anticipated that its total market opportunity will grow faster in the near future (greenbiologics, 2014).

2.3.3 Butanol production

Currently, butanol is mainly synthetic and is produced through a petrochemical route. Therefore, the production costs of synthetic butanol are mainly dependent on the market of crude oil. It can also be produced via ABE (acetone-butanol-ethanol) fermentation process. This biological process, due to use of renewable biomass including industrial and agricultural wastes as feedstock, is environmentally friendly. However, employing this bioprocess mainly depends on

the availability of abundant and cheap feedstock (Cascone, 2008; Dürre, 2007). As a result, if bio-butanol can be produced from renewable resources, and in an economically feasible way, it can be a replacement for bio-ethanol and bio-diesel in the bio-fuel market, assessed to be $247 billion by 2020 (Green, 2011).

2.4 Bio-butanol production: Brief history

To produce bio-butanol, usually ABE (acetone-butanol-ethanol) fermentation is involved. The ABE fermentation is one of the first large-scaled industrial processes, which can produce bio-butanol by fermentation of carbohydrates, and using solvents-producing strains of Clostridium (Kalil et al., 2003).

The ABE fermentation process was first used in the UK in 1912, in order to produce acetone for munitions. It was rapidly accepted for commercial production during World War I and II. After the end of World War II, due to the easier and cheaper petrochemical production of solvents, the bio-based solvent production was no longer cost effective, and this process became inefficient. Therefore, the fermentation process started to experience a decline, and by the 1960's, it was almost stopped in the U.S (Marlatt et al., 1986).

Recently, research into the ABE fermentation process received much more interest and researchers hope to improve fermentation process and bring bio-butanol back to the bio-fuels market (Figure 2-8).

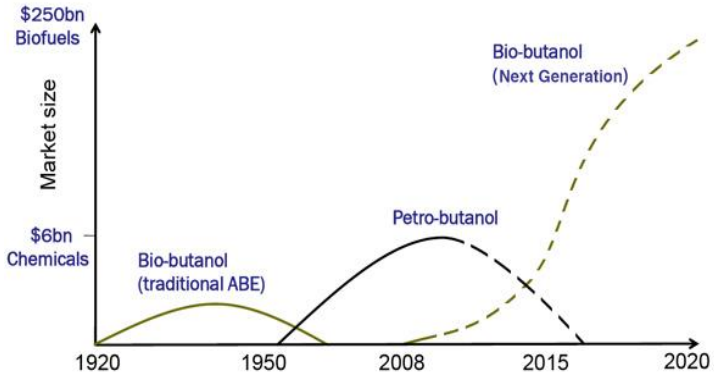

Figure 2-8 : Trends in butanol product ("Greenbiologics," 2014)

2.5 Bio-butanol production from hemicelluloses: Process

As discussed earlier, butanol, in addition to the chemical pathways, can be produced through biological methods under anaerobic conditions. The Clostridia spices are anaerobic microorganisms that was discovered by Haim Weizmann (Weizmann et al., 1937)and are well known for butanol production (Szulczyk, 2010). However, just a few types of Clostridium are able to use cellulose and hemicelluloses as a fermentation feedstock (Szulczyk, 2010).

As compared to the chemical methods of butanol production, the biological process has several advantages such as: ability to use the renewable resources (e.g. switch grass, wheat and corn core), higher product selectivity, and less formation of by-products. Furthermore, the operation conditions of butanol production through biological methods are milder than that of chemical processes, and the product separation is easier.

On the other hand, the pulp and paper industry is struggling with one of the toughest crises of its history. Therefore, to remain viable, the attempts should be focused on making the Canadian pulp and paper mills able to increase their revenue. The bio-refinery concept by production of large spectrum of value-added bio-fuels and chemicals from agricultural and forestry biomass, employing new technologies, and penetrating new markets can offer an opportunity to stimulate the Canadian pulp and paper industry and make it more competitive (Marinova et al., 2009).

A hemicelluloses extraction step is usually integrated in Kraft mill with dissolving pulp production, prior to pulping. This extracted hemicelluloses can be used as a feedstock to produce promising and valuable products such as xylitol, furfural and bio-fuels (e.g. bio-butanol and bio-ethanol) (Hu et al., 2008).

Recently, several studies have focused on investigating the economic profitability, and technical feasibility of butanol production from extracted hemicelluloses (Tunc et al., 2008).

Basically, the conversion of lignocelluloses to bio-butanol (Figure 2-9) consists of the following steps (Chegini et al., 2013b; Lynd et al., 2002)

- Pre-treatment / Pre-hydrolysis: To make the material more susceptible and accessible for degradation in order to release the hemicelluloses contained in the material (hemicelluloses extraction);

- Hydrolysis: To transform the hemicelluloses to fermentable sugars (in some research, the pre-hydrolysis and hydrolysis processes are integrated, and these processes are performed in one stage, and presented as pre-treatment, pre-hydrolysis or hydrolysis step) (Liu et al., 2013);
- Detoxification: To remove the fermenting toxic materials formed during pre-hydrolysis and hydrolysis;
- Fermentation: To convert the fermentable sugars to bio-butanol;
- Product recovery: To separate the butanol;

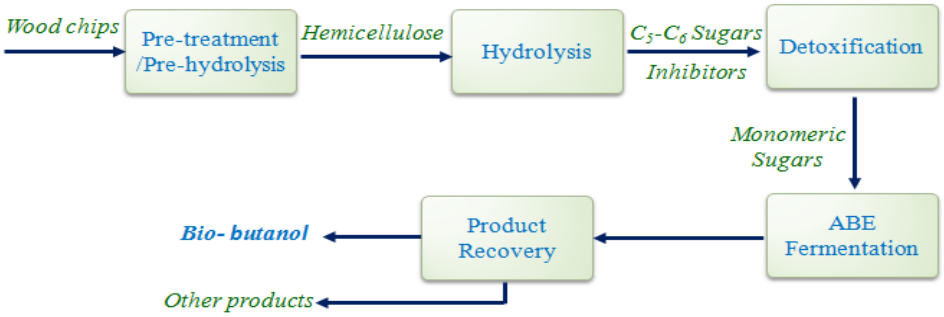

Figure 2-9 : Bio-butanol production from biomass
(Chegini et al., 2013b)

2.5.1 Pre-treatment/ Pre-hydrolysis

The first step of butanol production from biomass is pre-treatment, which in some references is also known as pre-hydrolysis.

In this step, the complex structure of lignocellulosic biomass is broken down (Figure 2-10) and with changing/removing the obstructing composition, the hydrolysis rate in further steps can be improved (F. Carvalheiro et al., 2008; Lynd et al., 2002). During the pre-hydrolysis process, the chains of hemicelluloses can be broken into oligosaccharides.

An efficient and cost-effective pre-hydrolysis method should be able to: minimize the formation of possible inhibitors, prevent the sugar loss and increase the hemicelluloses recovery. In addition, it should has low energy requirements, as well as low demand of subsequent processes such as washing and detoxification (Kumar et al., 2009; Zheng et al., 2009).

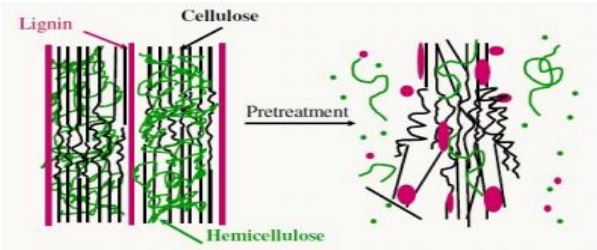

Figure 2-10 : Schematic of effects of pre-treatment process
on lignocellulosic biomass structure (Mosier et al., 2005)

There are several pre-treatment methods, which are divided into different categories including physical, chemical, biological or a combination of them (Kumar et al., 2009). These pre-treatment processes are summarized in Table 2-4.

Table 2–4 : Pre-treatment processes for lignocelluloses
Adopted from (Szczodrak and Fiedurek 1996, Szulczyk 2010)

Pre-treatment Method	**Operation resulting changes in the structure of substrate**	**Main changes**
Physical	Milling and grinding (pressure, hammer, ball and ball), high temperature (steam explosion and pyrolysis), irradiation (microwaves and electron beam), auto-hydrolysis;	Reduction in crystallinity and polymerization degree of cellulose, partial degradation of lignin and hydrolysis of hemicelluloses;
Chemical	Alkalis, acids, organic solvent, gases, reducers, oxidizers;	Delignification, decrease of crystallinity and polymerization degree of cellulose;
Biological	White-rot fungi (Pycnoporus, Phlebia, Pleurotus, Ischnoderma, etc.)	Delignification and reduction in degree of polymerization of cellulose and hemicelluloses;
Combined	Grinding followed by acid or alkaline treatment, Alkali-pulping associated with steam explosion;	Degradation of hemicelluloses, delignification

Usually, the hot water pre-treatment, due to its advantages such as limited equipment corrosion, less operational and capital cost, and lower cellulose break down receives much more attention. In addition, water is the main reagent in this method, which makes this process more economical and environmentally friendly than other pre-treatment methods (Tunc et al., 2008).

Another often used technique for pre-hydrolysis of lignocellulosic biomass is steam explosion, during which the biomass is treated with vapor under high pressure and temperature, and a rapid decomposition is performed. This causes the explosion of the biomass structure and its degradation , which facilitates the hydrolysis process (Peng et al., 2012, Lu, 2013).

2.5.2 Hydrolysis

As discussed earlier, during pre-hydrolysis of lignocellulosic biomass, the chains of hemicelluloses polysaccharide can be broken down into oligosaccharides and then can be hydrolysed to monosaccharides (fermentable sugars).

In order to perform the biomass hydrolysis process, several technologies have been employed including acid, enzymatic and alkaline hydrolysis. The performance and process conditions of different hydrolysis processes are compared in Table 2-5.

Table 2–5 : Comparison between dilute-acid and concentrated acid hydrolysis (Mohammad, 2008; M. J. Taherzadeh et al., 2007)

Hydrolysis Process	**Conditions**	**Advantages**	**Disadvantages**
Concentrated acid	30-70 % H_2SO4 T = 40 ºC Time = 2-6 h	- Operated at low temperature - Higher sugar yield - High reaction rate	-High acid consumption -High cost and energy consumption for acid recovery -Longer reaction time -Corrosion and environmental problem - Inhibitor formation
Dilute acid	1-5 % H_2SO_4 T = 215 ºC Time = 3 min	- Low acid consumption - Short residence time - High sugar recovery - High reaction rate	-Operated at high temperature - Equipment corrosion - Low sugar yield -Inhibitor formation
Alkaline	18 % NaOH T = 100 ºC Time = 1 h	High reaction rate	-Low sugar yield -Sugar decomposition by alkali attack
Enzymatic	T = 100 ºC Time = 1.5 day	-High yield of relatively pure sugar -Mild operating conditions -No environmental and corrosion problems	-Pre-treatment of biomass required -High cost of cellulose enzymes - Low hydrolysis rate

In acid hydrolysis techniques, dilute acid hydrolysis and concentrated acid hydrolysis are used. Sulphuric acid is commonly employed, although other acids such as HCL have been applied as well (Dehkhoda, 2008; Kumar et al., 2009).

The dilute acid hydrolysis process can be operated at high operating conditions with relatively short reaction time. Generally, during this process, the degradation of lignocellulosic biomass into sugars occurs. In general, hemicelluloses, due to its lower degree of polymerization (DP) and its branched structure are more sensitive to hydrolysis than cellulose. Therefore, the degradation of hemicellulosic sugars (mainly pentose sugars) is faster than cellulose-based sugars (six carbon sugars). By applying hydrolysis process in two stages, the yields of sugar conversion can be improved. The first stage is operated under moderate operating conditions to obtain the pentose sugars, while the recovery of hexoses can be optimized in the second hydrolysis stage (Mohammad, 2008).

The concentrated acid process is performed in relatively moderate conditions, with a much longer reaction time (Anish et al., 2009; Kumar et al., 2009). Similar to the dilute acid hydrolysis, the hemicelluloses break down into monomeric sugars, and provide the carbon source in the fermentation process (Lee et al., 1999).

The main challenges of the acid hydrolysis are their negative environmental effects, corrosion problems and high operating costs. Furthermore, during acidic hydrolysis, in addition to the fermentable sugar formation, some other by-products can also be generated. These by-products can be acetic acid (derived from acetyl groups), sugar products (furfural or hydroxymethylfurfural), and lignin derived compounds (Larsson et al., 1999). They usually obstruct the further bioconversion of monomeric sugars into desired products, and decrease the yield of fermentation step. This inhibitor formation can be considered as one of the main drawbacks of acidic hydrolysis.

Besides acids, the enzymes can also be used to degrade the hemicellulosic polymers into simple sugars at low temperatures. The enzymes with this ability are mostly known as hemicellulase (in general, the hemicellulase enzymes are classified under cellulase enzymes) (Smith, 2007). The moderate operating conditions, low by-product generation, and non-adverse reaction medium make the enzymatic hydrolysis a common process of lignocelluloses conversion to bio-fuels. However, compared to other hydrolysis techniques, some factors such as longer required

retention time, high cost of enzymes and low hydrolysis rate can limit the usage of the enzymatic hydrolysis process on industrial scale (Cardona Alzate et al., 2006; Kumar et al., 2009). The biomass hydrolysis can also be performed by employing an alkaline solution. Compared to other hydrolysis techniques, alkaline hydrolysis needs lower temperature and pressure, and can be performed at ambient conditions. However, the yield of sugar recovery is relatively low (Mohammad, 2008).

In this work, due to advantages of dilute acid hydrolysis this method was considered as hydrolysis method. The typical sugar profiles in acidic hydrolysates generated from hardwood are shown in Table 2-6.

Table 2–6 : Average sugar profiles of hardwood hemicelluloses acidic hydrolysates; (Nigam, 2001)

Sugar	**Concentration (g/L)**
Xylose	26.7 ± 1.30
Glucose	3.0 ± 0.18
Galactose	1.7 ± 0.02
Arabinose	1.5 ± 0.03
Mannose	6.5 ± 0.22

2.5.3 Detoxification

2.5.3.1 **Formation of inhibitors**

The dilute-acid treatment is a quick and inexpensive method to generate sugar from lignocelluloses (Larsson et al., 1999). However, the main disadvantage of this process is the formation of various by-products. Some of these compounds could have toxic effects on the cell growth, and decrease the yield and productivity of fermentation (Larsson et al., 1999; Zhuang et al., 2009). The formation of inhibitors is due to the following reasons:

- Some inhibitors may exist in the raw materials that are simply released during the pre-hydrolysis/hydrolysis treatments. An example is the phenolics, which are originating from lignin.
- Other inhibitors (e.g. acetic acid originating from acetylated hemicelluloses) can be found as side groups on the hetero–polymers, cut off during the pre-hydrolysis /hydrolysis steps.
- Some inhibitors such as furfural and HMF are formed during carbohydrate degradation.

The pre-hydrolysis and hydrolysis treatments may result in further degradation of lignin and monomeric sugars to three major groups of compounds that may inhibit the fermentation step. The inhibitors can be divided into three main groups (Figure 2-11): furan compounds including furfural and HMF derived from sugars, phenolic components (e.g. p-hydroxybenzaldehyde and vanillin) that are lignin derivatives, and finally weak acids (e.g. acetic, formic and levulinic), which can be formed from degradation of sugars (Palmqvist et al., 2000).

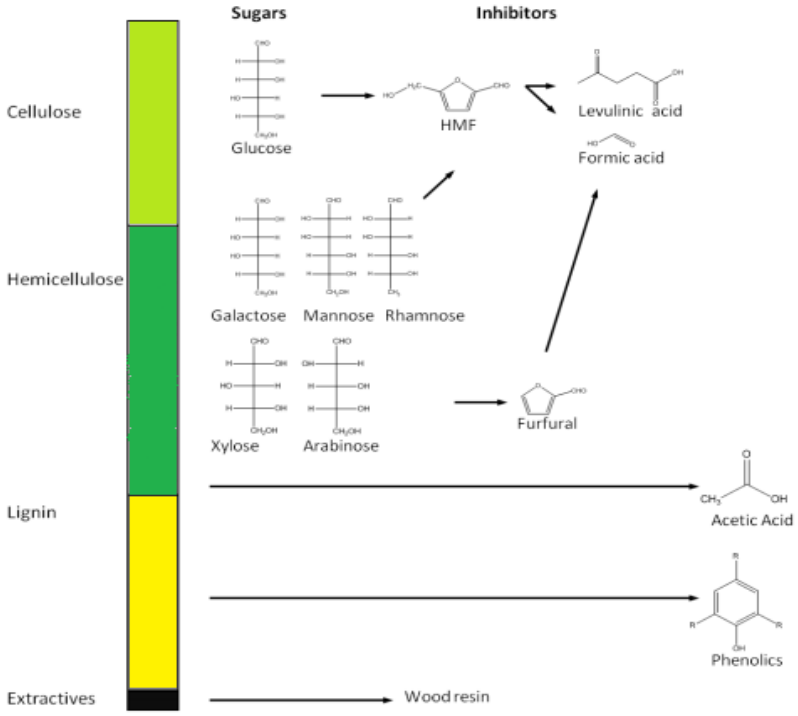

Figure 2-11 : The structural profile of fermentation inhibitors derived from lignocellulosic biomass with acidic treatment (Almeida et al., 2007; David Lukas Grzenia, 2011)

The inhibition of microbial growth and fermentation yield is specific (Table 2-7) both for each inhibitor and each microorganism. It mainly depends upon the type of fermenting organism, inhibitor concentration, cultivation conditions (e.g. temperature and pH), and cultivation mode. As it can be seen in Table 2-7, for any given microorganism, the toxic concentration for different inhibitors may vary. However, in most cases for the *clostridium* genus (the applied organism in present study), the following inhibitors are reported in order of reducing toxicity.

Phenolic compound > Furans > acetic acid

Table 2–7 : The toxic effects of inhibitors on some microorganisms

Candida guilliermondii FTI 20037	Acetic acid in concentrations higher than 3g/L can inhibit xylose metabolism	(Felipe et al., 1995)
Clostridium. beijerinckii BA101	Furfural (2 g/L) and HMF (1 g/L) do not have inhibitory effects on microorganism. However the mixture of them affects the culture negatively. Also C. beijerinckii BA101 is not inhibited by acetates; instead higher solvents are produced by the culture under high Concentrations of acetate in the medium.	(Ezeji, Qureshi, & Blaschek, 2007)
Clostridium beijerinckii NCIMB 8052	1 g/L of phenolic compound (e.g., p-coumaric acid, ferulic acid, 4-hydroxybenzoic acid, vanillin acid, syringaldehyde and vanillin) inhibited cell growth by 64 -74%, while entirely inhibiting the butanol production.	(Cho, Lee, Um, Sang, & Kim, 2009)
Clostridia acetobutylicum ATCC824	100 mg/L vanillin (phenolic compound) decreased the butanol yield from 0.19 gg^{-1} xylose to 0.046 gg^{-1}.	(Sun & Liu, 2012)
Clostridium acetobutylicum	By addition of 3.7 ~ 9.7 g/L acetic acid, the ABE concentration increased slightly, while it drastically reduced in the presence of 11.7 g/L acetic acid. But, the solvent production of C. beijerinckii was not affected by addition of acetic acid in the range of 3.7 ~ 11.7 g/L.	(Cho, Shin, & Kim, 2012)
P. stipitis	In the presence of 2 g/L furfural and 3 g/L acetic acid, no sugar consumption was reported and cellular growth was completely inhibited.	(Chaud, Silva, Mattos, & Felipe, 2012; Díaz et al., 2009)

Detailed descriptions of acidic treatment derived inhibitors are presented in the following parts.

2.5.3.1.1 Organic acids

The most common existent weak acids in lignocelluloses hydrolysates are acetic acid, levulinic acid and formic acid. The levulinic and formic acids are derived from HMF breakdown, while acetic acid is mainly produced from acetyl groups in the hemicelluloses.

Formic acid can additionally be generated from furfural at high temperature and under acidic conditions. There are also various types of fatty acids including 9, 12-octadecadienoic and hexadecanoic. In addition, there are some branched aliphatic acids such as methyl botanedioic acid, methyl propanedionic acid and 2-methyl-2-hydroxybutanoic acid. These compounds, due to their low concentrations, do not have significant effects on the microorganism's metabolism (Luo et al., 2002; Purwadi, 2006). It is well demonstrated that the acetic acid toxicity is dependent on pH (Gottschalk, 1986). Acetic acid is a weak acid and unlike strong acids, doesn't dissociate completely. The non-dissociated acids have negative effects on the cells and can hinder the cell growth. They are liposoluble compounds that can penetrate the cell membrane, and may dissociate inside the cell, where the pH is nearly neutral. In order to keep intracellular pH stable and take out the excess protons (H+ ions), the cell consumes energy (Luo et al., 2002; M. J. Taherzadeh et al., 2007). Hence, at a high concentration of un-dissociated acids (higher than critical extracellular concentration), the transport rate of protons might exceed the diffusion capacity of membrane and intracellular acidification can happen, which results in cell death or cell growth hindering (Larsson et al., 1999; Palmqvist et al., 2000). The microorganism tolerance to acetic acid is case specific, and mainly depends on the microorganism and operating conditions. Taherzadeh represented the extracellular pH limit of yeast at several concentrations of acetic acid. It was observed that the yeast was able to grow in existence of 10 g/L acetic acid at its extracellular pH limit (higher than 4.7). Therefore, it was demonstrated that acetic acid is harmless if the cultivation is performed at a pH level not less than extracellular pH limit, or if its concentration is low (M. J. Taherzadeh et al., 1997). However, Maiorella observed the negative effects on microbial growth and an ethanol yield in as low as 0.25 g/ L of acetic acid (Maiorella et al., 1983).

2.5.3.1.2 Phenolic Compounds

The inhibitors derived from lignin are phenolics, polyaromatic compounds and aldehyides; it is considered that their toxicity is proportional to the molecular weight. It was found that phenolics

with lower molecular weight exhibit stronger toxic effects on microorganisms than those with higher molecular weight (Palmqvist et al., 2000). In lignocellulosic hydrolysates, there are several phenolic compounds, which have been detected such as ferulic acid, 3-methoxy-hydroxybenzaldehyde, vaniline, vanilic acid acetovanilone, 4-hydroxyacetophenone, and 4-hydroxybenzoic acid (Luo et al., 2002); the type of generated phenolics mainly depends on the applied biomass (Klinke et al., 2003).

The phenolic compounds, are one of the inhibitors with high toxic effects (Larsson et al., 1999). It has been observed that low molecular weight phenolics can limit the cell growth of *S. cereviseae* in the fermentation process (Larsson et al., 1999). In addition, it has been shown that about 1 g/L of 4-hydroxybenzoic acid caused about 30% reduction in the ethanol yield in comparison with the control fermentation. It has also been reported that inhibition of fermentation was reduced, when phenolic compounds were specially eliminated from a willow hydrolysate (Jönsson et al., 1998).

The phenolic components break up the biological cell membrane and cause integrity damage. Therefore, the membrane ability to act as a selective barrier can be disturbed (Luo et al., 2002; Parawira et al., 2011). However, their inhibition mechanism has not been clarified yet (Luo et al., 2002; Parawira et al., 2011).

2.5.3.1.3 Furan compounds

Furans, including furfural and 5-hydoxymethyl furfural, are a major group of inhibitors. The level of furans compounds based on the source of substrate and the applied pre-hydrolysis/ hydrolysis methods can differ. For example, the concentration of HMF in spruce hydrolysate may vary from 2.0 g/L to 5.9 g/L related on, whether the dilute acid hydrolysis is carried out in one step or two steps (Almeida et al., 2007).

Furfural has been reported to have a strong inhibitory effect on *S. Cerevisiae*. Concentrations above 1 g/L of furfural significantly reduce the cell multiplication, the rate of CO_2 evolution, and the total number of viable cells in the preliminary fermentation phase (Boyer et al., 1992; Parawira et al., 2011; Sanchez et al., 1988; M. J. Taherzadeh et al., 1997). During aerobic fermentation, furfural is oxidized to furoic acid, while during an anaerobic cultivation; furfuryl alcohol can be generated from furfural reduction. It is believed that alcohol dehydrogenase is

responsible for furfural transformation in both cases (Luo et al., 2002; Palmqvist et al., 2000; M. J. Taherzadeh et al., 1997).

Due to the structural similarity of HMF and furfural, their inhibitory effects are similar. However, the experimental results showed that in comparison to HMF, furfural has stronger toxic effect on fermentation and growth of *S. cerevisiae*. It was also shown that adding 4 g/L of HMF reduced the specific growth rate with 70%, the CO_2 evolution rate with 32%, and the ethanol production rate with 40% (M. Taherzadeh et al., 2000), while the same amount of furfural showed a stronger inhibitory effect. (M. Taherzadeh et al., 2000).

2.5.3.2 **Detoxification Methods**

As discussed earlier, biomass hydrolysis produces not only monomeric sugars but also different compounds, which may have inhibitory effects on the microbial metabolism and sugar conversion to the bio-fuels. In some cases, the existence of one compound can increase the inhibition of other compounds on the microorganism's metabolism. As an example, this synergetic toxicity demonstrated that the formation of acetic acid and furfural can cause an undesirable effect on cell mass production, growth rate and ethanol production yield of *S.cerevisiae* (Palmqvist et al., 2000). Thus, it is necessary to eliminate inhibitors to a minimal level. Moreover, it should be considered that the composition of inhibitor components depends on the source of lignocellulosic biomass, the employed pre-hydrolysis/ hydrolysis methods, and the applied operational conditions (e.g. pressure, time, pH and temperature). This variety resulted in development of different detoxification methods, which can be chemical, physical or biological (David Lukas Grzenia, 2011). In table 2-8, several detoxification techniques that have been reported in literature to remove toxic components from biomass hydrolysates are compared. However, due to the different toxicity tolerance levels of various microorganisms, comparing the efficiency of the detoxification methods is not easy. Essentially, each method is efficient in removing specific inhibitors. Some methods reduce the sugar contents, which results in lower bio-fuel yield. In some cases (e.g. poplar hydrolysate) the combination of two or more detoxification processes may increase the ethanol production (Cantarella et al., 2004). However, an economic evaluation had not been carried to show if the combination of two detoxification process is practical on an industrial scale (David Lukas Grzenia, 2011).

Table 2–8 : Comparison of different detoxification methods to remove inhibitors from lignocellulosic hydrolysates

Process		Advantages	Disadvantages	References
Physical Methods				
Evaporation/ Concentration	Removing inhibitors by evaporation in a vacuum concentrator based on the volatility	Reduces volatile compounds such as acetic acid, furfural, and vanillin	-Increasing the non-volatile inhibitors as extractives	(Anish et al., 2009) (Mussatto et al., 2004)
Membrane	Membranes have surface functional groups attached to their internal pores, which may eliminate metabolic inhibitors	Avoids the need to disperse one phase and minimize the entrainment of small amounts of organic phase	- High cost - Selective removal of inhibitors	(Anuj K Chandel et al., 2011; David L Grzenia et al., 2012)
Chemical/ Physiochemical Methods				
Activated Charcoal	Adsorption of toxic compounds by charcoal, which is activated to increase the contact surface	-Low cost - Remove phenolics and furans - Low sugar loss	Filtration complexity	(Mussatto et al., 2004) (Canilha et al., 2004)
Overlimming	-Increase of the pH followed by reduction - Precipitate toxic compounds	- High sugars loss	- Filtration complexity	(Palmqvist et al., 2000)
Ion Exchange Resin	Resins change undesirable ions of the liquid phase to be purified by saturating of functional groups of resins	- Remove lignin-derived inhibitors, acetic acid and furfural - Low sugars loss - can be regenerated and reused	- High pressure - Long processing time - Possible degradation of fragile biological product molecules - hard to scale-up	(Canilha et al., 2004) (Anuj K Chandel et al., 2011) (Carvalho et al., 2004) (Nilvebrant et al., 2001)
Extraction with Organic Solvents	Mix of liquid phase to be purified with an organic solvent. The liquid phase is recovered by separation of two phases (organic and aqueous)	-Recycling of solvents for consequent cycles - Remove acetic acid, furfurall ,vanillin and low molecular weight phenolics	-High cost - Long processing time	(Wilson et al., 1989) (Cantarella et al., 2004)
Biological Methods				
Microorganism	Specific enzymes or microorganisms that act on the inhibitors compounds present in hydrolysates and change their composition	- Low waste generation - Environmentally friendly - Less energy requirements	Long processing time	(Anish et al., 2009), (Anuj K Chandel et al., 2011), (Hou-Rui et al., 2009),(Yang et al., 2008)

In Table 2-9, non-biological detoxification methods, which have been applied to different lignocellulosic hydrolysates, are summarized. As it can be observed, each method represents its

specificity to remove certain inhibitors from the hydrolysate. Based on the global assessment of the published results, the effectiveness of reported detoxification methods to remove common inhibitors is shown in Table 2-10.

Table 2–9 : Different non-biological detoxification methods applied to lignocellulosic hydrolysates

Lignocellulose Hydrolysate	**Detoxification methods**	**Changes in hydrolysate Composition**	**References**
Aspen	Roto-evaporation	Removal of acetic acid (54%), furfural (100%) and vanillin (29%)	(Wilson et al., 1989)
Oak wood	Activated charcoal	Removal of phenolics (95.40%)	(Converti et al., 1999)
Sugarcane bagasse	Neutralization	NA	(Anuj Kumar Chandel et al., 2007)
Corn stover	Membrane based organic phases alamine 336	60% acetic acid removal	(David L Grzenia et al., 2008)
Wheat straw	Ion exchange-D 311 + over-liming	Removal of furfurals (90.36%), phenolics (77.44%) and acetic acid (96.29%)	(Zhuang et al., 2009)
Wheat straw	Ethyl acetate + over-liming	Removal of furfurals (59.76%), phenolics (48.23%) and acetic acid (92.19%)	(Zhuang et al., 2009)
Spruce wood	Dithionite and sulfite	No major change in composition of hydrolysates	(Alriksson et al., 2011)
Saccharum spontaneum	Over-liming	Removal of furfurals (41.75%), total phenolics (33.21%), no effect on acetic acid content. Reduction of reducing sugars (7.61%)	(Anuj K Chandel et al., 2011)
Sugar Maple	Nano-membrane	removal nearly all small molecular organic acids (acetic acid, formic acid), furfural and HMFand and slightly less than 50% of phenolics	(Sun et al., 2012)

Table 2–10 : Effectiveness of non-biological detoxification methods
(Chegini et al., 2013b)

	Inhibitors		
Detoxification method	**Furans**	**Weak acids**	**Phenolics**
Over liming	√	X	√
Activated Charcoal	X	X	√√
Evaporation	√√	√	√
Nano-membrane	√√	√√	√
Ion Exchange	√	√	√

In this study, due to the capability of membranes to ensure high purity and energy savings, the membrane separation was proposed as detoxification process. Another advantage of using membrane in the current study is the concentration of pre-hydrolysate.

2.5.4 Membrane filtration: Basic principles

Membrane is a layer of material, which can be employed as a selective barrier between two homogenous phases. There are two main phases that are usually considered in membrane filtration: concentrate (upstream side) and permeate (downstream side). As shown in Figure 2-12, when the membrane is exposed to the action, some specific molecules or particles are rejected by the membrane and accumulate in the concentrate stream, while others are allowed to pass through the membrane and go into the permeate stream (Gutman, 1987; Mohammad, 2008; Mulder, 1996). This transportation can occur because of differences in chemical and physical characteristics between the permeating substances and membranes.

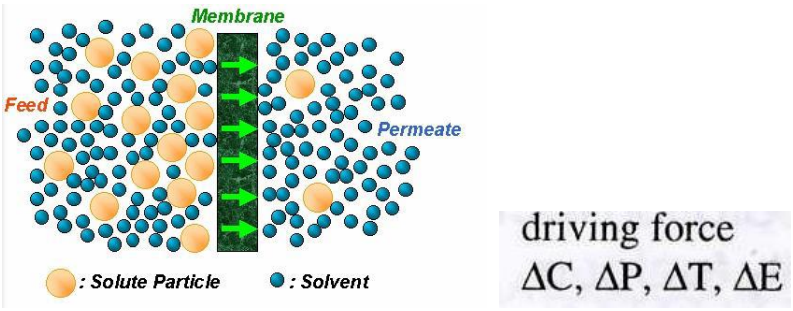

Figure 2-12 : Schematic of membrane separation (Mohammad, 2008)

Essentially, the membrane filtration process, based on the module configuration and membrane application, can be categorized into two different types: dead-end flow filtration and cross flow filtration.

2.5.4.1 **Dead-end filtration**

During a dead-end filtration process, the fluid passes through the membrane and the particles/molecules larger than the membrane pore size can be retained at the membrane surface (Figure 2-13). This means that the trapped molecules start to form a "filter cake" on the membrane surface, which decreases the filtration process efficiency until elimination of the filter cake (Rautenbach et al., 1989).

2.5.4.2 **Cross/ Tangential flow filtration**

The cross flow filtration is carried out based on the flowing of a feed solution along a membrane surface. In this process, the portion of fluid passes through the membrane and goes into the permeate side by applied pressure, while the retained molecules are washed and removed by the tangential flow, consequently the accumulation of particles on the membrane surface can be prevented (Figure 2-13).

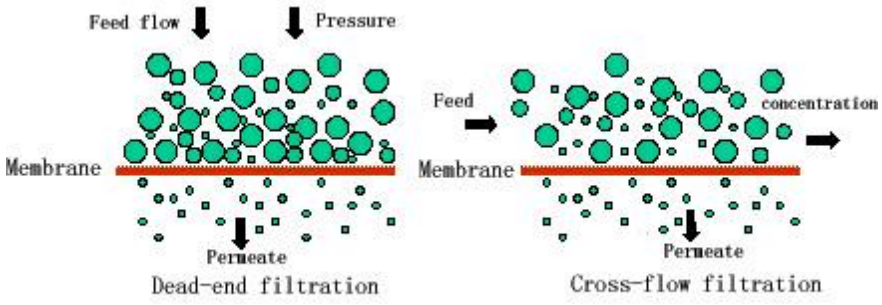

Figure 2-13 : Types of filtration process (Bilad, 2010)

Membranes are classified in several categories: they might be thin or thick, hydrophilic or hydrophobic; their structure can be heterogeneous or homogenous (Mulder, 1996). Furthermore, the transportation through membranes can be active (in which the movement from areas with lower concentration to areas with higher concentration needs chemical energy), or passive (in

which the movement is from high concentration areas to low concentration areas, and there is no need to apply energy). The driving forces in a membrane with passive transportation is the difference in concentration, pressure, temperature or electrical potential (Mohammad, 2008; Rautenbach et al., 1989).

Typically, a membrane separation can be applied for purification or concentration purposes (Gutman, 1987). In addition, this process is efficient in continuous mode and easy to combine with other separation processes. Moreover, the membrane filtration can be scaled up simply. However, fouling and flux reduction, short lifetime of the polymeric membrane, and in some cases inadequate selectivity can be considered as drawbacks (Mulder, 1996; Rautenbach et al., 1989). Key factors in membrane separation are: the membrane structure, the size of solute particles, and the chemical characteristics of solute (Mulder, 1996).

The efficiency and performance of membrane separation can be described by two main parameters: membrane selectivity and flow through membrane. The membrane selectivity is mainly expressed by two parameters, which are separation factor (α) and retention factor (R) factor. The retention factor (rejection factor) is usually employed to describe the solute selectivity in aqueous solutions, while the separation factor is applied for the cases of gas mixtures (David L Grzenia et al., 2012; Gutman, 1987; Mulder, 1996). The flow parameter is also expressed by the term permeates flux (permeation velocity), which is specified as the volume that passes through the membrane per unit area and time.

During a pressure driven membrane filtration, the separation efficiency often varies with the time, and the typical behavior of permeate flux reduction may occur (Mohammad, 2008). This phenomena can happen because of several factors such as fouling (placing of solutes particles at the membrane surface or inside the membrane pores), gel layer formation, and concentration polarization[3](Affleck, 2000; David L Grzenia et al., 2012; Mulder, 1996). These factors induce

[3] Concentration polarization is the dynamic accumulation of rejected feed solids at the membrane surface, which may occurs because of selective transport of some molecules through the membrane under the effect of driving forces (Porter, 1972). The result of this accumulation is formation a boundary layer near the surface of membrane, which is mainly referred to as the "gel layer". Gel layer can be noticed as the super saturation of retained species, which can result in localized gel layer near the surface of membrane (Adams, 2012).

extra resistances for permeate passage through the membrane, and consequently decreases the permeate flux (Mohammad, 2008). Typically, the effects of concentration polarization and fouling (pore blocking) behaviors strongly depend on the feed solution and the membrane type (Gutman, 1987; Mulder, 1996). These effects, which are more intensive in the pressure driven membranes, can be significantly limited by applying the cross-flow (tangential flow) filtration.

2.5.5 Pressure driven membrane /Process

In the pressure driven membrane processes, the driving force is the pressure difference. The pressure applied on the feed at one side of the membrane operates as a driving force, and results in separation of the feed solution into the permeate and concentrate phases (Van der Bruggen et al., 2003).

Pressure driven membranes are generally classified into four main groups: micro-filtration, ultra-filtration, nano-filtration, and reverse osmosis. During these filtration processes, by applying pressure, the solvent and some small solute molecules pass through the membrane and go to the permeate side, while the other particles or molecules are rejected by the membrane and are retained on the concentrate side. The rates of solute rejection and solvent permeation mainly depend on the solute molecule size and the membrane structure. The particle size or molar mass of the molecules that can be permeated, reduces from microfiltration through ultra-filtration and nano-filtration to reverse osmosis (Gutman, 1987). The smaller pore size results in higher mass transfer resistance through the membrane. This resistance enhancement indicates that in order to get the same flux value, significant driving force has to be applied (Gutman, 1987; Mulder, 1996).

The pressure driven membranes are generally defined by the nominal pore size and molecular weight cut-off (Unit: Dalton). Each group rejects specific molecules and their major differences are the pore size and molecular weight cut-off (Cheryan, 1998).

The molecular weight cut-off (M.W.C.O) is a characteristic, which is mainly employed by the membrane producers to specify the retention capacity of the membrane and normally refers to the lowest molecular weight (in Dalton) of a solute (e.g. protein and dextrin), where the membrane has a solute retention of higher than 90%. In other words, the smallest particles can pass through the membrane and form the permeate solution, while the larger entity (where rejection is >90%)

are "cut-off" from the permeate stream (von Recum, 1998). The membrane pore size is also described by the diameter of the particles, which are expected to be rejected by a membrane with a specified degree of efficiency. The pore size is commonly presented in micrometer (µm), which is one millionth of a meter (Cheryan, 1998). The main principles of the pressure driven membrane filtration processes are summarized in Figure 2-14.

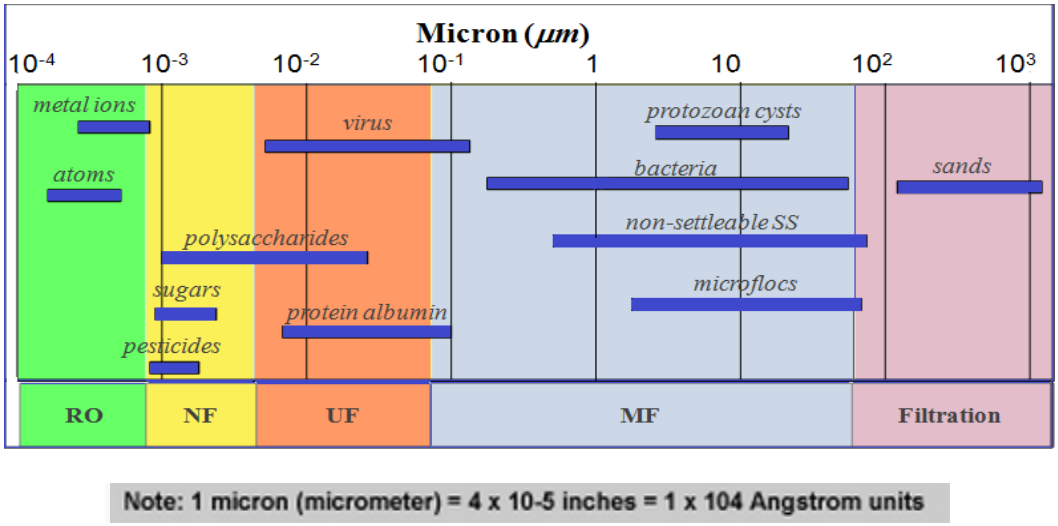

Figure 2-14 : Ranges of membrane based separation
(Chegini et al., 2013b; Peter, 2011)

A detailed description of the pressure-driven membranes is given in the following sections. Their separation characteristics are also presented in Figure 2-15.

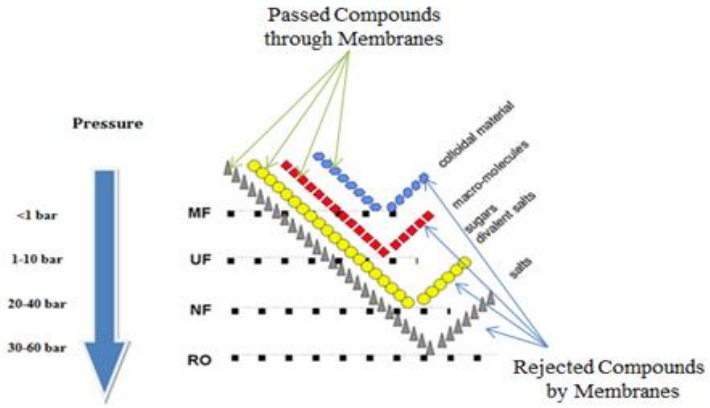

Figure 2-15: Separation characteristics of pressure driven membranes;
Adopted from (Affleck, 2000; Cheryan, 1998)

2.5.5.1 **Microfiltration (MF)**

This membrane process is a low-pressure process to separate and retain the suspended solid materials with particle size of ~0.1 to 10 µm or larger, while the smaller particles (e.g. sugars, salts, sugars and proteins) can pass through the membrane. The sieving mechanism is the separation principles, and the filtration process is usually based on the size and shape of the solute particles (Gutman, 1987; Mulder, 1996).

By applying cross flow velocity, the effects of fouling, concentration polarization or gel layer on the membrane surface can be reduced. These parameters might result in a permeate flux reduction, as compared with the pure water flux (Gutman, 1987; Mohammad, 2008; Mulder, 1996). In addition, the regular membrane cleaning can be noted as a method to prevent the flux reduction. Therefore, the membrane materials have to be resistant to the applied cleaning method (Mohammad, 2008). Several inorganic (e.g. TiO2 and Al_2O_3) and polymeric materials (e.g. polyamide) are usually employed to make microfiltration membranes. (Affleck, 2000; Mohammad, 2008; Mulder, 1996). Microfiltration can be applied for the concentration, clarification (e.g. fruit juice) and wastewater treatment. In addition, cold sterilization of pharmaceuticals and beverages can be considered as another possible application of microfiltration.

2.5.5.2 **Ultra-filtration (UF)**

Ultra-filtration is a medium-pressure method offering retention of macromolecules, such as polymers and proteins, in addition to the particles including colloids, emulsions and biological materials. The range of molecule size, which can be retained by ultra-filtration membranes is approximately 0.01 – 0.1 µm. The organic solvents with low molar mass (e.g. salt ions, and other small solute molecules) can easily pass through the ultra-filtration membrane. The applied driving pressure is usually lower than 10 bars (Gutman, 1987; Mohammad, 2008; Mulder, 1996).

The filtration principles of micro-filtration and ultra-filtration are similar, and are based on the sieving mechanism. However, the hydrodynamic resistance of MF membranes is lower than UF membranes. The common polymers, which are used to prepare the UF membranes, are polyethersulfone and polysulfone (Affleck, 2000).

The main parameter to choose an appropriate UF membrane for concentration of an identified macromolecular solution is the molecular weight cut off, which is a number presented in g/mol showing that in most cases, 90% of the molecules with higher molar mass will be rejected by the membrane (Mohammad, 2008; Mulder, 1996).

The main problems in the UF separation are fouling and concentration polarization phenomena. These behaviors can happen due to the solute concentration and the accumulation of the rejected macromolecular solutes on the membrane surface. This problem can be solved by applying the cross flow filtration, in which the feed flows tangentially over the surface of the membrane rather than inside the membrane (dead-end filtration). In this method, the filter cake, which can block the membrane pores, is washed away and removed (Mohammad, 2008; Rautenbach et al., 1989).

The major usage of UF membrane is as a filtration stage in the food and dairy industries. Ultra-filtration process has also some applications in metallurgy and in drinking water purification (Gutman, 1987; Mohammad, 2008).

2.5.5.3 **Nano- filtration (NF)**

Nano-filtration refers to a separation process in order to retain the micro-organic pollutants (e.g. insecticides and herbicides) and the organics with low molar mass in the range of 100-1000 g/mole. In fact, the NF membranes usually do not have practical pores, but only free available spaces inside the polymeric network. The typical operating pressure in a NF process is between 20-40 bar (Gutman, 1987; Mulder, 1996; Rautenbach et al., 1989).

Usually, NF membranes are manufactured commercially by companies such as Millipore (Bedford, Mass) and Osmonics (Minatanka, MN)(Affleck, 2000).

The industrial usages of nano-filtration membrane are the concentration of sugars, divalent salts, enzymes, bacteria, proteins, antibiotics and dyes, and other molecules with molecular weight higher than 1000. The hard water softening can also be considered as another possible application of NF membranes (Affleck, 2000; Gutman, 1987; Mohammad, 2008; Mulder, 1996; Rautenbach et al., 1989). In addition, the multivalent salts such as calcium salts can be rejected and retained by NF membranes.

2.5.5.4 **Reverse Osmosis (RO)**

Osmosis phenomenon is an automatic flow of pure water (in most cases) from low to high concentrated aqueous solution, where a semi-permeable membrane is employed as a separation barrier. The reverse osmosis process forces water to flow through the membrane from a high concentrated aqueous solution to a lower one. In this process, membranes with extremely fine pores and made from cellulose acetate are utilized. The diameter of these pores is typically less than 0.001 micron (μm). However, in the case of RO, such fine pores cannot be observed with a microscope (Mulder, 1996).

Reverse osmosis is a high-pressure process that can retain almost all low molar mass solutes and ionic species. Operating pressure is ranging from 30 to 60 bars, which may exceed ~76 bars in some cases (Mohammad, 2008).

The reverse osmosis process can be applied in seawater desalination, where the high drinkable water recovery can be achieved from seawater in a one-step operation. Moreover, by applying RO membranes, the retention of solvents with low molar mass (e.g. methanol and ethanol) can be performed efficiently (Rautenbach et al., 1989).

In the current study, in addition to the membrane concentration, in order to get higher product purity, a dia-filtration process was proposed as a complementary deotixification stage.

2.5.6 Dia-filtration

Dia-filtration is a separation process with the aim of "washing out" the permeable particles. This filtration process can also be used to increase purity. In dia-filtration, water is added into the feed reservoir, while permeate is eliminated continuously. In this process, where the products of interest are in the concentrate, other components are washed out and removed from the concentrate phase into the permeate phase. This can result in a reduction in concentration of undesirable components.

In order to minimize the amount of additional water and consequently reduce the time and the cost of dia-filtration process, it is sometimes better to concentrate the product before subjecting it to the dia-filtration step(s). Concentration refers to the volume reduction of the solution at the beginning of the process. It can be done by collecting permeate without adding any water to the process vessel. Because the particles with larger molecule size are rejected by the membrane, as a

result the concentration of those particles will increase. However, the concentration of permeable molecules remains unchanged.

Figure 2.16 shows a 2x-concentration, i.e. when the concentration of the rejected particles doubled, the process volume decreases by half.

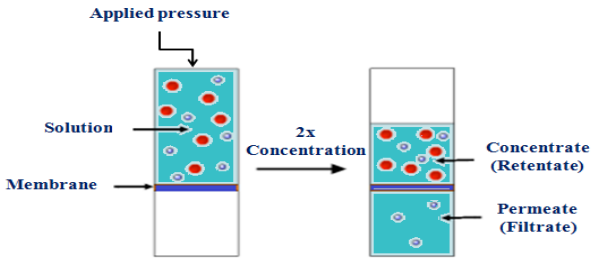

Figure 2-16 : 2x-concentration of a mixed sample[4]; adopted from (Schwartz, 2003)

2.6 ABE Fermentation

Acetone-Butanol-Ethanol (ABE) fermentation is a process that produces acetone, butanol and ethanol from renewable resources by using solvent-producing strains. In a typical batch ABE fermentation, due to the product inhibition, the maximum solvent production (acetone, butanol and ethanol) does not go higher than 20 g/L, of which the butanol concentration is about 8-13 g/L (Cascone, 2008; Jones et al., 1986). This low product concentration is one of the main challenges of the ABE process and results in low productivity (<0.6 g/L·h) and low yield (~0.3 g/g sugar), which consequently may cause the high costs of product recovery during downstream processing (Qureshi et al., 2001).

The common technical and commercial challenges for the ABE fermentation are presented in Table 2-11(Green, 2011).

[4] Larger circles represent particles, which are bigger than the membrane pores and small circles represent molecules, which are smaller than membrane pores.

Table 2–11 : The drawbacks and solutions for improving ABE fermentation

Drawbacks	**Solutions**
High feedstock cost mainly leads to high operating costs.	Switch towards more sustainable and cheaper sources (e.g. wastes and agricultural residues).
Butanol inhibition and low butanol titres enhance the costs of recovery as well as water usage.	Develop improved microbes with improved solvent titres and/or develop techniques for in situ product recovery to decrease butanol inhibition.
Low volumetric productivity of solvent increases the operational and capital costs.	Develop continuous fermentation processes in order to increase volumetric productivity and decrease operational time.
Low butanol yield enhances feedstock costs.	Develop improved strains with a higher butanol yield and/or develop microbes with higher ratio of butanol to solvent.
Solvent separation employing conventional distillation is energy intensive and expensive.	Develop low energy methods for product recovery.
High water usage is not sustainable and increases the cost of waste treatment.	Recycle water back through the fermentation.
The possibility of inhibitor formation during previous steps (mainly acidic treatment)	Apply a suitable detoxification process

The current study tried to address some drawbacks of the ABE process by using a cheap feedstock and reducing the concentration of inhibitors.

2.6.1 Microorganism

ABE fermentation is an anaerobic process, in which the weight production of acetone, butanol and ethanol is in a ratio of 3:6:1, respectively. This process generally uses the clostridia class of bacteria such as *Clostridium acetobutylicum* and C*lostridium beijerinckii* as solvent- producing microorganisms. In recent decades, several strains of these two species have been widely studied, including *C. acetobutylicum ATCC 824, P260, P262*, as well as *C. beijerinckii ATCC 55025*, and *BA101* (Chang, 2010; Peng et al., 2012; Qureshi et al., 2001 ; Tashiro et al., 2007). However, ATCC824 is a well-known strain and due to its potential for solvent production, its application in the ABE fermentation is receiving attention.

In this study, the bacteria of *Clostridium acetobutylicum ATCC 824* is applied in the fermentation tests.

2.6.2 Microbial growth

In general, microbial growth can be explained as an orderly enhancement of cellular components and consequently cell enlargement, which finally leads to cell division.

The typical growth curve (Figure 2-17) of a batch culture process has the following phases (Aleksic, 2009):

- Lag phase: the delay before beginning of the exponential growth and the cells are adapting to the new environment;
- Exponential phase: where cell division occurs at a constant rate;
- Stationary phase: in which bacteria stop replicating because of unfavorable conditions for growth;
- Death phase or decline phase: when cells lose their viability;

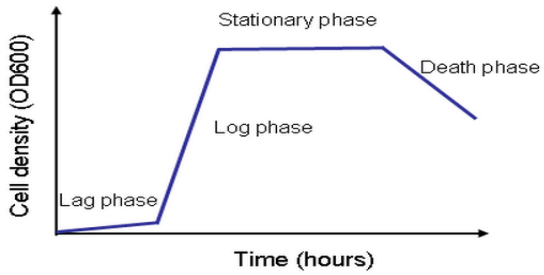

Figure 2-17 : Schematic bacteria cell growth curve (Inc., 2003)

In the case of ABE fermentation, the use of *Clostridium* results in two separate characteristic stages: acidogenesis and solventogenesis (Chang, 2010; Tashiro et al., 2007):

- Acidogenic phase: the initial phase in which the cell growth is exponential and due to the enhancement of acid contents, a reduction in pH occurs.
- Solventogenic phase: by acid accumulation and pH reduction to < 5.0, a metabolic shift to the solventogenesis phase is induced. During this stage, cell growth enters the stationary phase and the microorganisms utilize the accumulated acid in the initial phase as an additional carbon source in order to produce acetone, butanol and ethanol (Tashiro et al., 2004, Tashiro, 2007).

Although, ABE fermentation process has been studied over several years, the mechanism of the metabolic shift from the acidogenesis phase to the solventogenesis phase is not still clear.

Overall, comprehensive studies on fermentation process, product separation and substrate pre-treatment are required in order to get higher butanol yield from the ABE fermentation, decrease the total production cost and make this process efficient and economically viable.

2.6.3 Media for fermentation

An important factor in fermentation is the use of a suitable medium, which mainly depends on the desired products and the type of microorganism (Aleksic, 2009).

Fermentation microorganisms need essential materials such as water, sources of carbon, energy, nitrogen, mineral elements and vitamins.

Essentially, an ideal medium, should be able to (Aleksic, 2009; Costa et al., 1983):

- Produce the maximum biomass per gram of substrate used;
- Generate the maximum product concentration;
- Minimize the formation of undesired products;
- Minimize the impact on sterilization process, product purification and waste treatment.

The main components of any fermentation process are water and energy. The energy for the microbial growth can mainly be provided from the light and medium components such as carbohydrates, lipids and proteins. The choice of carbon source depends on the microorganisms applied in the fermentation process, as well as the main final products. (Aleksic, 2009; S.-M. Lee et al., 2008; Sun et al., 2012).

In terms of nitrogen, most industrially used microorganisms can utilize both organic and inorganic sources of nitrogen. Organic nitrogen can be supplied as amino acid, urea or protein, while inorganic nitrogen may be provided as ammonia gas, nitrates or ammonium salts. All microorganisms also need specific mineral elements for their growth and their metabolic activity. In most media, sulphur, potassium, calcium, chlorine, magnesium and phosphorus are considered as the essential mineral components (Aleksic, 2009).

2.7 Product recovery

Distillation is often used for butanol separation. However, due to the low average concentration of butanol at the end of the ABE fermentation process (~10 g/L – 20 g/L), the butanol recovery by employing distillation method is not economically viable.

In order to reduce the cost of butanol separation, the final butanol concentration should be enhanced. Several techniques have been used to recover butanol from the fermentation reactor and obtain high concentration. These techniques are liquid-liquid extraction, gas stripping, and pervaporation (Chang, 2010; S. Y. Lee et al., 2008).

Gas stripping is an easy and effective method for butanol separation (Fig 2-18, a). Butanol is removed by bubbling fermentation gas through the fermentation broth and the butanol containing gas passes through a condenser to recover condensed butanol. The stripped gas is sent back to the bioreactor and the fermentation process continues until all the sugars are consumed. By removing butanol from the fermentation broth, due to reduction of product inhibition, the rate of sugar consumption and consequently the final butanol yield are enhanced.

The liquid-liquid extraction is another effective way for solvent separation from fermentation broth (Figure 2-18, b). In this technique, the butanol is recovered by employing an extractant, which is immiscible with the fermentation broth. Butanol is more soluble in organic phase (extractant) than in aqueous phase (fermentation broth). Therefore, it can be separated, and selectively concentrated in the extractant. This extracted solvent can be later recovered by distillation. Liquid-liquid extraction can be used if the extractant is cheap, insoluble in the fermentation broth and non-toxic to the cells. The selected extractant should also be able to extract solvent selectively (Ezeji et al., 2004). Oleyl alcohol and decanol are the most common extractants (S. Y. Lee et al., 2008).

Another common method of butanol recovery is pervaporation, which is a membrane-based method and can selectively separate volatile compounds from the fermentation broth (Figure 2-18, c). The volatile liquid or solvent diffuses through the membrane, and can be recovered during transmission through a condenser. The separating membrane can be applied in the form of solid or liquid with supporting substances (S.-M. Lee et al., 2008).

Each of these three separation techniques has its own benefits and drawbacks. However, despite the progress in the yield and productivity of the ABE fermentation by employing the in situ product recovery processes, the butanol and total solvent yields still need improvement.

Moreover, the integration of product recovery step could affect the fermentation process. As an example, the bubbles in gas stripping might generate more shear stress, which would be harmful for the bacterial cells. In the case of pervaporation and liquid-liquid extraction, finding the proper extractant or materials with the essential physical and chemical specifications is not simple on an industrial scale (Kraemer et al., 2011; S. Y. Lee et al., 2008).

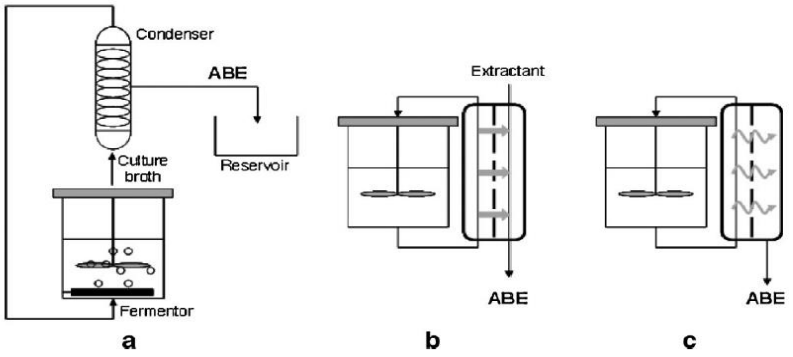

Figure 2-18 : Integrated processes for fermentation and in situ product recovery: fermentation combined with (a) gas stripping, (b) liquid–liquid extraction and (c) pervaporation (S. Y. Lee et al., 2008)

2.8 Summary of literature review

A hemicelluloses extraction step can be integrated in Kraft pulp mills prior to the pulping, and the hemicellulosic sugars can be further converted into value added products, such as bio-fuels. Butanol, due to its advantages can be considered as a suitable fuel alternative. However, the conversion of hemicellulosic sugars into bio-butanol suffers from several challenges; one of them is the generation of toxic materials prior to fermentation. In order to decrease the negative effects of the inhibitors, the use of an efficient detoxification step(s) before fermentation step is required. The focus of this study was the concentration and detoxification steps prior to fermentation. Membrane separation, due to its advantages (e.g. energy saving), was proposed as a detoxification method and its performance in eliminating inhibitors from hemicellulosic hydrolysates was studied.

To limit the problem related to membrane fouling, the filtration processes were performed in cross flow filtration mode. Moreover, in order to get higher concentration of sugars, applying the dia-filtration step in combination with the concentration process was proposed.

In the final step of this work, the microbial growth of *C. acetobutylicum* ATCC824 on different samples (including non-detoxified and concentrated-detoxified pre-hydrolysate and hydrolysates) was investigated.

Chapter 3 OBJECTIVES & METHODOLOGICAL APPROACHES

3.1 Main Objective

To study the effect of membrane separation on the removal of potential inhibitor compounds from hemicelluloses hydrolysates in order to produce bio-butanol (bio-fuel).

3.2 Specific Objectives

The specific objectives of this work were:

- To evaluate the performance and efficiency of five different commercial membranes , including nano-membrane and ultra-membrane, to eliminate the inhibitors and retain the sugars.
- To investigate the performance of dia-filtration combined with concentration processes for sugar concentration and inhibitor removal.
- To evaluate the performance of membrane separation in two different scenarios: concentration after hydrolysis and concentration before hydrolysis.
- To study the growth profile of the butanol-producing microorganism, *Clostridium acetobutylicum,* on detoxified - concentrated hemicelluloses pre-hydrolysate and hydrolysate.

3.3 Methodology

In this work, two main scenarios were studied. The first scenario was performing the concentration process after hydrolysis step, which is the common method in bio-fuel production. Furthermore, due to the potential advantages of pre-hydrolysate concentration prior to hydrolysis, the second scenario, which was concentration before hydrolysis, was also suggested. The potential benefits of this scenario are:

- Reducing base utilization in the neutralization step which will result in less salts formation;
- Decreasing acid utilization in the hydrolysis step;
- Saving more energy for solution heating.

3.3.1 Concentration after hydrolysis (Scenario 1)

The main experimental processes in the first scenario are:

- Step 1. Pre-hydrolysis
- Step 2. Acid hydrolysis
- Step 3. Detoxification
    - Membrane selection
    - Concentration followed by two steps dia-filtration with the selected membrane
- Step 4. ABE fermentation

3.3.2 Concentration before hydrolysis (Scenario 2)

The main experimental steps in the second scenario are summarized as follows:

- Step 1. Pre-hydrolysis
- Step 2. Detoxification
    - Membrane selection
    - Concentration of pre-hydrolysate followed by one step dia-filtration with the selected membrane
- Step 3. Acid hydrolysis
- Step 4. ABE fermentation

The steps of the methodology are detailed in the following sections.

3.4 Contributions

In this work, the efficiency of nano-filtration and ultra-filtration to concentrate and purify the hemicellulosic hydrolysates, which was obtained from mixed maple and aspen wood chips, was investigated. To the best of our knowledge, there was no report on treatment of the mentioned hydrolyzates (mixed maple-aspen) by using the membranes that are examined in the current research. However, some studies on detoxification of hemicellulosic hydrolysates from other sources of biomass with similar organic content have been reported. Moreover, the membranes tested in this study (pressure driven membranes), due to their relatively low energy requirement,

high selectivity, and the possibility of applying them in a broad temperature range without the need of chemicals addition, have potential to gain popularity in industrial applications.

This research uses the concept of "waste to wealth" owing to utilization of hemicelluloses as cheap and abundant substrate to yield butanol, which due to its advantages compared to ethanol, is a suitable alternative to replace it.

In addition, detoxification by using membrane separation, which is suggested in this research, compared to conventional chemical processes, has various advantages such as higher efficiency, easy scale up and no emulsion generation. Essentially, the membrane technology, as an energy-saving separation and a highly selective process, has a great potential to be employed in the bio-refinery and bio-fuel production industry. Therefore, the results of the present work make progress in bio-fuel production industry:

- Sugar concentration;
- Detoxification of hemicellulosic hydrolysate prior to fermentation;
- Potential production of higher value-added products (e.g. bio-ethanol and bio-butanol) by an optimal choice of membrane pore size;
- Employing cheap and abundant feedstock in the Canadian Pulp and Paper industry and provide a competitive advantage to this industry.

Chapter 4 MATERIALS AND ANALYSIS METHODS

4.1 Raw Materials

4.1.1 Pre-hydrolysate

In this study, the pre-hydrolysate of mixed hardwood chips, consisting of maple and aspen from a Canadian dissolving pulp mill, was used. This pre-hydrolysate was kindly provided by FPInnovations.

4.1.2 Microorganism

In the fermentation step, the *Clostridium acetobutylicum ATCC824,* which is the most commonly applied strain for ABE fermentation (Ramey et al., 2004), was employed as the solvent-producing microorganism. This strict anaerobic microorganism is saccharolytic bacterium, which is able to ferment a broad variety of sugars, oligosaccharides and polysaccharides, and convert them to solvents (Stim-Herndon et al., 1996).

In this work, the *C.acetobutylicum ATCC824* was obtained from the American Type Culture Collection and the stock cultures of this strain were maintained in 10% (vol /vol) glycerol at −80 °C.

4.1.3 Growth medium (culture medium)

In current work, the growth of *C. acetobutylicum ATCC 824* was studied in two different growth mediums. The first medium was Reinforced Clostridial Medium (RCM), which is an enriched and non-selective medium formulated by Hirsch and Grinstead (Hirsch et al., 1954). This medium was expanded for isolation and cultivation of anaerobic spore-forming microorganism specifically Clostridium species (L'USO). The second medium was the synthetic medium, which based on the literature could be considered as a suitable medium for clostridia growth (Peng et al., 2012). These mediums were composed of the following components (per liter of distilled water):

- Reinforced Clostridial Medium (RCM): Peptone(10 g), Beef Extract(10 g), Yeast Extract (3 g), Xylose (60 g), Agar (0.5 g), Sodium Chloride (5 g), Soluble Starch (1 g), Cysteine HCl (0.5 g), Sodium Acetate (3 g);
- Synthetic Medium (SM): Yeast extract (1 g), CH_3COONH_4 (2.2 g), KH_2PO_4 (0.5 g), K_2HPO_4. $3H_2O$ (0.5 g), $MnSO_4$ (0.01 g), NaCl (0.01 g), $MgSO_4.7H_2O$ (0.2 g), $FeSO_4.7H_2O$ (0.01 g), Biotin (0.01 mg), P- aminobenzoic acid (1 mg) and xylose (60 g).

4.2 Experimental equipments

4.2.1 Membranes

Two major separation experiments were conducted in this study: membrane selection and detoxification (concentration and dia-filtration).

In the membrane selection step, the main objective was to select a suitable membrane that could retain sugars and polysaccharides, while allow inhibitors to pass through the membrane. Based on the specifications of pressure driven membranes and their filtration spectrum (Fig 2.13), the ultra- filtration and nano-filtration, due to their potential specifications for sugar concentration and inhibitor removal, were selected for further studies. The performance of five types of commercially available membranes, including three nano-membranes (NF90, NF270 and XN45) and two membranes for ultra-filtration (UE10 and UA60), with various nominal molecular weight cut-off (M.W.C.O) was studied. As discussed in membrane separation part, the molecular weight cut-off is a characteristic that normally should be determined empirically by the membrane manufactures, and is most commonly employed to identify nano-filtration and ultra-filtration membranes (Cheryan, 1998; von Recum, 1998).

According to the membranes manuals that were provided by manufactures, the main characteristics of the used membranes are represented in Table 4-1.

Table 4–1 : Main specification of tested membranes

Model**	**Process**	**Membrane Material**	**M.W.C.O (Da)***
DOW FILMTEC™ **NF90**	Nano-filtration	Polyamide	200
DOW FILMTEC™ **NF270**	Nano-filtration	Polyamide	400
4040-**XN45**-TSF	Nano-filtration	Polyamide	500
4040-**UA60**-TSA	Ultra-filtration	Polyamide	1000-3500
4040-**UE10**-QSF	Ultra-filtration	Polyethersulphone	10000

* Molecular Weight Cut-Off (Dalton)

** The maximum operation temperature and pressure for all tested membranes were 45 °C and 4.1 MPa respectively.

4.2.2 Filtration experimental set up

In the current study, a lab-scale flat-leaf testing unit (SEPA CF II, GE-Osmonics), with an effective area of 140 cm^2, was used to perform the filtration experiments. This system also contained a hydraulic hand pump membrane (SPX POWER TEAM) to pressurize the cell holder (Fig 4-1).

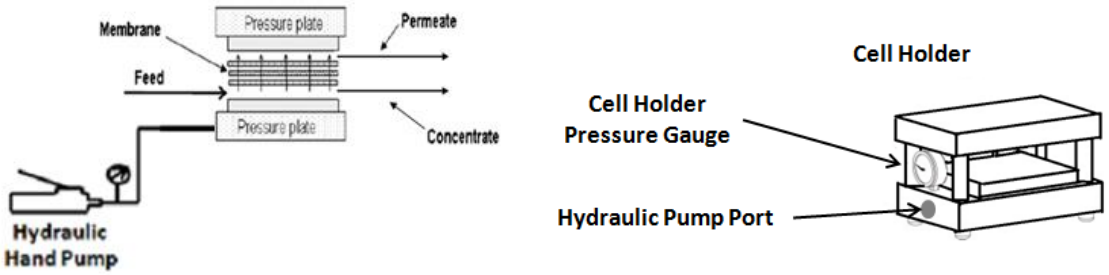

Figure4-1 : Schematic of Osmonics flat-leaf test apparatus with a membrane (Chegini et al., 2013a)

In the filtration unit, by using a positive displacement pump (hydra-cell), the feed was pumped from the feed tank with volume of 4L to the membrane cell. This pump provided the trans-membrane pressure in the system.

As mentioned before, in this study, the membrane filtration experiments were divided in two main parts. The first part was membranes selection, in which the system was performed under

total recirculation mode and both permeate and concentrate streams were collected in the feed tank (Fig 4-2). The second part was a detoxification step including concentration and dia-filtration processes. In this part, the permeate streams were collected in different tank, while the concentrate stream flowed back to the feed vessel (Figure 4-3). The filtration experiments were carried out in the temperature less than 40 °C, which was recommended by membrane manufacturers. Furthermore, in order to limit the membrane fouling, all the separation experiments were performed in the flow filtration mode.

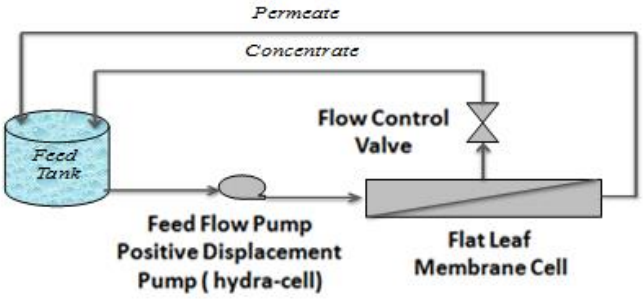

Figure 4-2 : Experimental set up for membrane selection phase
(Chegini et al., 2013b)

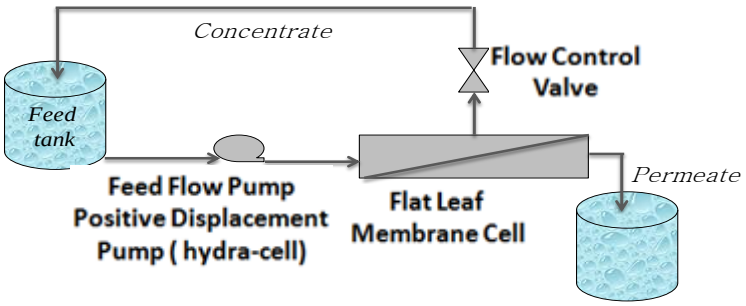

Figure 4-3 : Set up used for detoxification (concentration and dia-filtration) phase
(Chegini et al., 2013b)

4.3 Analysis methods

The analyses were conducted in duplicate and in some cases in triplicate.

4.3.1 Total solids

The samples of pre-hydrolysate and hydrolysate were analyzed for total solids by drying the 25 g of samples at 105 °C for 24 hr. These samples, before recording the weight of evaporating dish and dry samples, were placed in desiccators to cool. The dry matter percentage was measured as the remaining weight of sample after drying, and expressed as the percentage of the wet sample (the received sample).

$$\%\text{Dry matter} = \frac{\text{Weight of dry sample}}{\text{Weight of wet sample}} \times 100 \qquad \text{Equation 1}$$

4.3.2 Reducing sugar

The reducing sugar concentration was determined by using the DNS (3, 5-dinitrosalicylic acid) method (Rivers et al., 1984). DNS reagent solution, which was prepared by dissolving 1 g of DNS reagent (by heating at 80 °C) in 20 ml NaOH (2M), was diluted with water to a final volume of 50 ml. Then 30 g of Potassium sodium tartrate (Rochelle salt) was added and diluted to 100 ml with distilled water. 2 ml of DNS reagent was added to each assay tube and these tubes were maintained in boiling water bath for 4 min. Then, the samples were cooled down to the room temperature before determination of their absorbance value at 570 nm (Gonçalves et al., 2010). The amount of reducing sugars that were released, was determined from d-glucose anhydrous (0.1% w/v) standard curve against absorbance at 570 nm by the equation $y = 6.5675x - 0.0091$ ($R^2 = 0.9993$).

4.3.3 Furfural, hydroxymethyl furfural (HMF) and organic acids

Furfural, HMF and organic acids were analyzed by HPLC (Agilent Technologies) with 210 nm diode array detector (DAD) and Supelco C18 (150 X 4.6 mm) column, using a temperature of 35 °C, 0.01 M potassium phosphate as the mobile phase, flow of 1 µl min-1 and sample volume of 20 µl.

4.3.4 Total phenol content

Total phenol content of samples was determined by using the Folin-Ciocalteu technique (Singleton et al., 1965). Briefly, the amount of 5 ml of each sample was added to a 35 ml of water, and was assayed with 2 ml of Folin reagent and 5 ml sodium hydroxide (6%, w/v). The mixture was diluted with water to a final volume of 100 ml. After 60 min incubation in dark and at the room temperature, the absorbance was read at 725 nm. The total phenols in the extracts were expressed as gallic acid equivalents (GAE) by using a calibration curve of a freshly prepared Gallic acid solution (Mraicha et al., 2010). For the gallic acid, the curve absorbance versus concentration was described by the equation $y = 0.0058x - 0.023$ ($R^2 = 0.9949$).

4.3.5 The pH and conductivity

In order to determine the ionic contents of the samples, their pH and conductivity were measured by using a Fisher Scientific–accumet (Model 25) pH meter and a Thermo-Scientific conductivity meter respectively.

4.3.6 Membrane efficiency

In filtration experiments, the efficiency of membranes for inhibitor removal and sugar retention was calculated as:

$$\text{Removal Efficiency (\%)} = C_{permeate} \times 100 / C_{feed} \qquad \text{Equation 2}$$

Whereas:

C_f: Solute concentration in the feed

$C_{p:}$ Solute concentration in permeate

4.3.7 Fouling

The irreversible membrane fouling is specified as the pure water permeability reduction after filtration process divided by the initial water permeability, as demonstrated in the following (Qi et al., 2012):

$$\text{Irreversible fouling (\%)} = \frac{\text{PWFb} - \text{PWFa}}{\text{PWFb}} \times 100\% \qquad \text{Equation 3}$$

Whereas:

PWFb: Pure water permeability before filtration

PWFa: Pure water permeability after filtration

4.3.8 Cell concentration (fermentation step)

In the fermentation step, in order to investigate the microorganism growth, the cell concentration was measured by the optical density (OD) at 600 nm and by using a Pharmacia Biotech Spectrophotometer (NOVASPEC II).

Chapter 5 EXPERIMENTAL WORK, RESULTS & DISCUSSION

5.1 Pre-hydrolysis/Hydrolysis

The pre-hydrolysate was generated by using steam and hot water. The debarked wood chips containing aspen-maple (~ 63% wt. solid content) were loaded into a 56-liter digester and purged with steam (3× 3 min) at 138 kPa. Later, the digester was sealed and steam was introduced to reach a temperature of 170 °C and a pressure of 700 kPa for 50 min. In order to maintain the digester at 170 °C for another 60 minutes, additional steam was used. In the next stage, deionised water was heated up to 80 °C, at the end of the steam pre-hydrolysis stage, 7 liters of hot water were pumped into the digester and the circulation pump was activated. Because of the water addition, the digester temperature dropped, and returning it back to 170 °C took 10 minutes. The circulation was continued for an additional 5 minutes. Finally, the pre-hydrolysate was collected and later submitted to the hydrolysis and filtration steps (Abril et al., 2012).

To convert the polysaccharides into sugar monomers, the pre-hydrolysate was hydrolyzed with 1.5 wt% sulfuric acid at 120 °C for 30 min; the pH was reduced to 3.05 from 3.31 (the pH of pre-hydrolysate). After neutralization with NaOH, the hydrolysate was filtered with a microfiltration membrane (pore size 1.5 µm) to remove the precipitate (Sun et al., 2012).

The samples of pre-hydrolysate and acidified hydrolysate were analyzed for sugars and inhibitors and the results were compared (Table 5-1 and Table 5-2). In terms of sugar content, the dominant sugar in the hardwood pre-hydrolysate was xylose (5-carbon sugar). After performing acid hydrolysis, the xylose concentration was increased to 33.4 g/L from 1.18 g/L (Table 5-1). The increase of xylose concentration in the hydrolysate was due to the conversion of the oligomeric sugars into monomers.

Table 5–1 : Sugar concentration in pre-hydrolysate and hydrolysate

Monomeric Sugars (g/L)	**Pre-hydrolysate**	**Hydrolysate**
Xylose	1.18	33.4
Manose	0.03	1.10
Glucose	0.01	0.09
Sucrose	0.07	0.11
Total	1.29	34.7

As shown in table 5-2, after performing acid hydrolysis, the concentration of inhibitors in the hydrolysate was increased.

In addition, the conductivity[5] of hydrolysate at 17.5 °C (room temperature) was measured and it was 20.8 δ/cm ± 0.5%, which was around 10 times higher than the conductivity of pre-hydrolysate at 16.5 °C (2.1 δ/cm ± 0.5%). This higher conductivity indicates more purity in hydrolysate, which was resulted from elimination of precipitated materials.

Table 5–2 : Specification of pre-hydrolysate and hydrolysate

Component	**Pre-hydrolysate**	**Hydrolysate**
HMF(mg/L)	49	57.6
Furfural(mg/L)	528.3	606.2
Phenolics (mg/L)	563.8	1415
Acetic acid(g/L)	2.34	5.37

[5] Siemens per meter (δ /m) is the unit of conductivity in SI. The conductivity of typical drinking water is in the range of 5-50 μS/m.

5.2 Detoxification: concentration after hydrolysis (scenario 1)

The applied methodology in the first scenario is shown in figure 5-1.

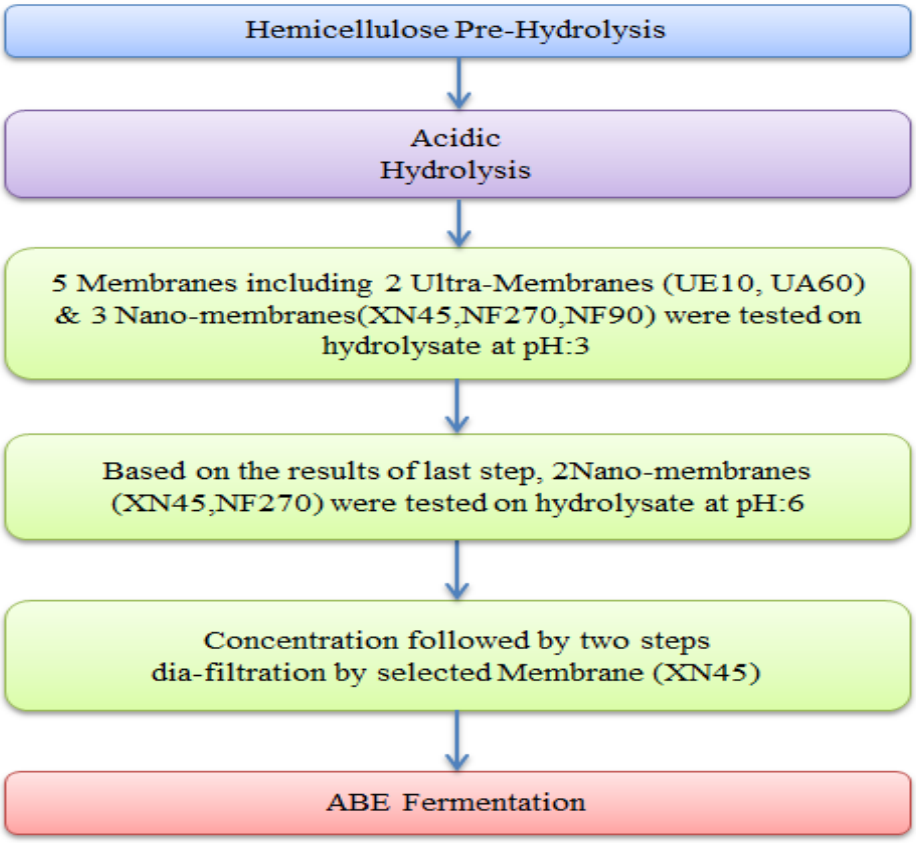

Figure 5-1 : The applied methodology in the first scenario (concentration after hydrolysis)

5.2.1 Membrane Selection:

After performing the pre-hydrolysis and hydrolysis steps, in order to study the membrane ability to remove the inhibitors and retain the sugars, the experiments were performed with the flat leaf cell unit in a cross flow filtration mode. These separation experiments were conducted by testing five selected membranes, including three nano-filtration membranes (NF90, NF270 and XN45) and two ultra-filtration membranes (UE10 and UA60) with various nominal molecular weight cut-off (M.W.C.O).

At the beginning and the end of each run filtration, in order to detect the intensity of the membrane fouling, each membrane was compacted with deionized water at 100-200 psi until a constant value of permeate flux was reached. During this process, the system was in a total recycle mode; in which both permeate and concentrate streams were collected in the feed tank (Figure 4-2). The hydrolysate solution was pumped through the system at one end, whereas the concentrate and permeate (filtrate) were discharged at another end. For each membrane, under steady-state condition and total recycle mode, the samples of the hydrolysate before filtration (Ai) and after filtration that was mixture of concentrate and permeate (Af) were taken. The permeate samples (5±1 mL) were also collected from time 0 to 90 min at an interval of 30 min (P0, P30, P60, and P90) and were analyzed in terms of sugars and inhibitors concentration. The results are presented in figures 5-2 and 5-3. The pH level was 3±0.1, which was the pH of the acid hydrolysate (the related tables are available in appendix A). In all the samples, the conductivity was measured immediately after their collection and in most cases, a significant reduction of conductivity was observed, which indicates higher purity.

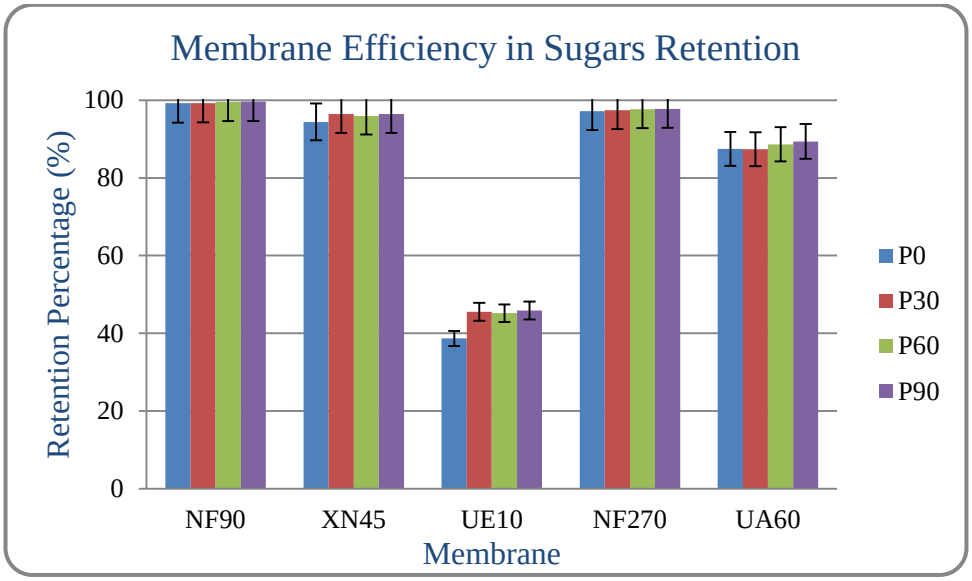

Figure 5-2 : Total sugars retention in hydrolysate by tested membranes at different intervals ; first scenario at pH 3 (error bars reflect the percentage errors).

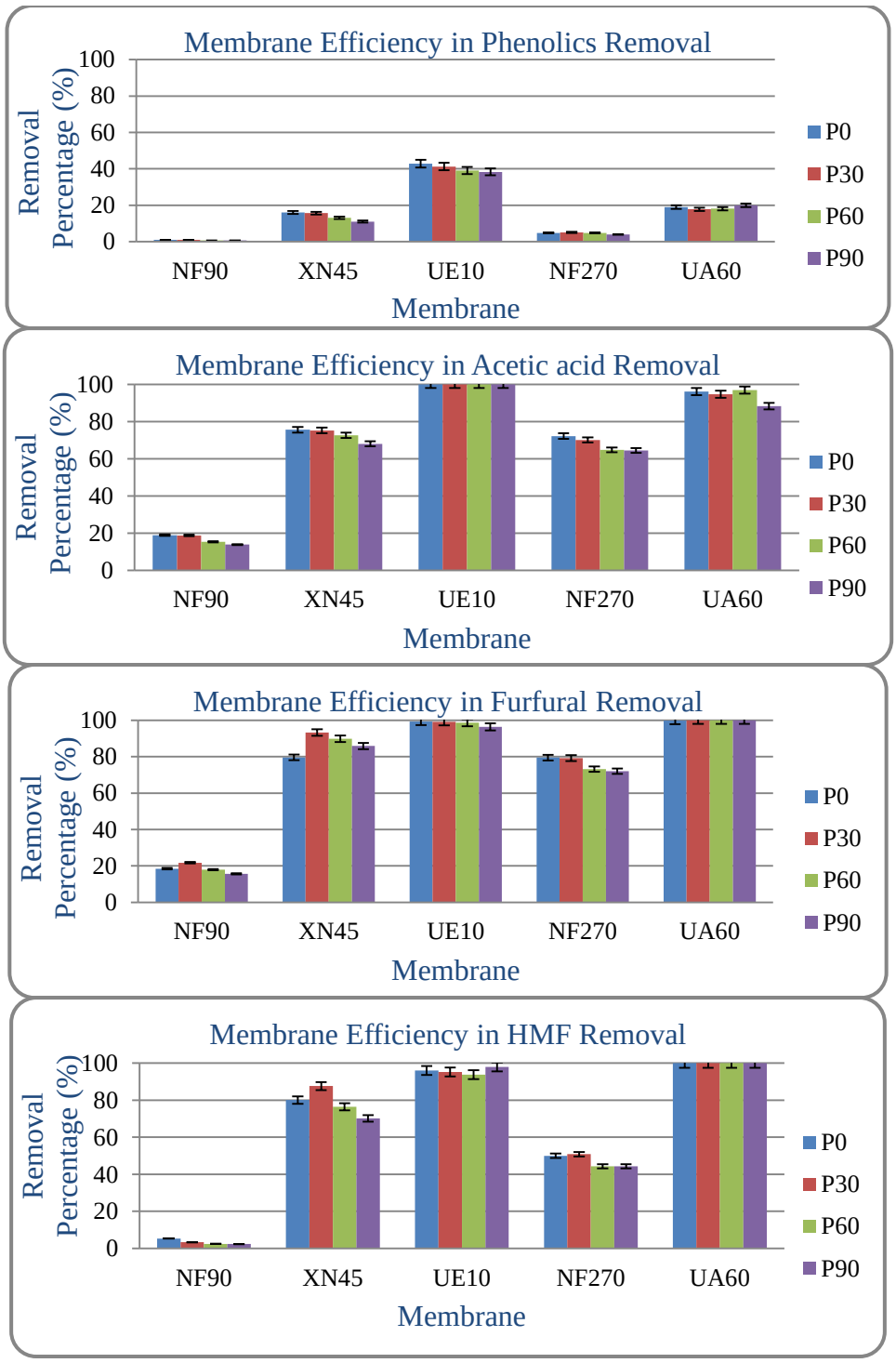

Figure 5-3 : Removal of inhibitors from hydrolysate
by means of membranes at different intervals ; first scenario at pH 3
(error bars reflect the percentage errors).

As it can be observed, in terms of sugars and inhibitors contents, the time did not have a significant effect on the obtained efficiencies (steady-state conditions), the analysis results of permeate samples (P0, P30, P60 and P90) were similar.

According to the average results, the molecular weight cut-off and efficiency of the examined membranes can be compared as follows:

Phenolic removal: $\mathrm{UE10} > UA60 > XN45 > NF270 > NF90$

Acetic acid removal: $\mathrm{UE10} > UA60 > XN45 > \simeq \mathrm{NF270} > NF90$

Furans (Furfural and HMF) removal: $\mathrm{UE10} \simeq \mathrm{UA60} > XN45 > NF270 > NF9$

Sugars loss: $\mathrm{UE10} > UA60 > XN45 > NF270 > NF90$

Molecular weight cut-off: $\mathrm{UE10} > UA60 > XN45 > NF270 > NF90$

The obtained separation results and comparing them with molecular weight cut-off of the tested membranes indicated that the higher molecular weight cut-off resulted in the higher removal efficiency of membranes, and in general, the membrane cut-off had a significant effect on the filtration performance.

Moreover, the membranes of UE10 and UA60 showed higher efficiency for elimination of potential inhibitors, and almost a complete removal of acetic acid, furfural and HMF was observed. However, the use of these membranes resulted in higher sugars loss (Figures 5-2 and 5-3). These higher inhibitors removal and lower sugars retention by UE10 and UA60 is due to their high molecular weight cut-off (as indicated in Table 4-1: 1000-3500 and 10000 Da respectively), which if compared to the molecular weight of the inhibitors components (Table 5-3), are high enough to let them pass through the membranes and be removed from the concentrate stream. However, the phonics removal, even in the membranes with high molecular weight cut off (e.g. UE10), was low. This behaviour can be explained by the hydrophobic nature of lignin derived phenolic compounds.

Table 5–3 : The molecular weight of potential inhibitors and sugars

Component	**Molecular Weight (g/mol)***
Acetic acid	60.05
Furfural	96.06
HMF	110.11
Pentose	150.13
Hexose	180.16
Phenolics	138 -194

*(1 g/mol = 1 Da)

Another affecting factor for the low phenolic removal percentage by UE10, is the limited adsorption capacity of the hydrophobic polyethersulfone structure of UE10 (Acero et al., 2005).

In terms of sugar retention, as can be noticed in Figure 5-2, NF90 was the most efficient membrane and offered the highest sugar retention (more than 98%). However, it had the least inhibitor elimination and due to its low molecular weight cut-off (Table 4-1), most of the substances with higher molecular weight, including potential inhibitors and sugars, were retained.

In our study, the concentrate streams were the solutions that were recovered and used in the further steps. Furthermore, the focus of membrane selection experiments was to find the suitable membrane that was able to remove more inhibitors and retain more sugars. Therefore, in other words, a membrane with lower inhibitors rejection (less inhibitor compounds in concentrate and more in permeate) and higher sugar retention (more sugars in concentrate and less in permeate) is required.

Taking into account that the NF90, UE10 and UA60, in the case of first scenario, were not able to meet both criteria at the same time, the two membranes of XN45 and NF270 were selected for further experiments.

In the next step, by using NaOH, the pH was adjusted to 6. The concentration of sugars, weak acids, furan derivatives, and phenolic compounds in feed and permeate streams were determined. Figures 5-4 and 5-5 present the removal percentage of sugar and inhibitor contents with tested membranes at different times at pH of 6 (The related tables are available in appendix A).

According to the results, at pH of 6, the membrane XN45 showed higher inhibitor removal than NF270. However, its sugar retention was slightly lower than NF270. Moreover, comparing the results of selected membranes (NF270 and XN45) at two applied pH indicates that after increasing the pH to 6, the maximum efficiency of NF270 and XN45 to remove phenolics did not

change significantly. However, by increasing the pH level from 3 to 6, the acetic acid removal percentage by NF270 had a reduction in an approximate range of 20-30%. This behavior can be described by dissociation effect. NF270 is a hydrophilic and polyamide-based nano-membrane, which has carboxylic groups at its surface (Artuğ, 2007; Mänttäri et al.). At higher pH, due to dissociation of the carboxylic groups, the surface obtains negative charges. On the other hand, when the pH level of a solution is higher than the acetitc acid's pKa (= 4.75 at 25°C), acetic acid would be dissociated and its anionic form could be rejected by a negatively charged membrane surface; consequently the removal percentage of acetic acid would be decreased.

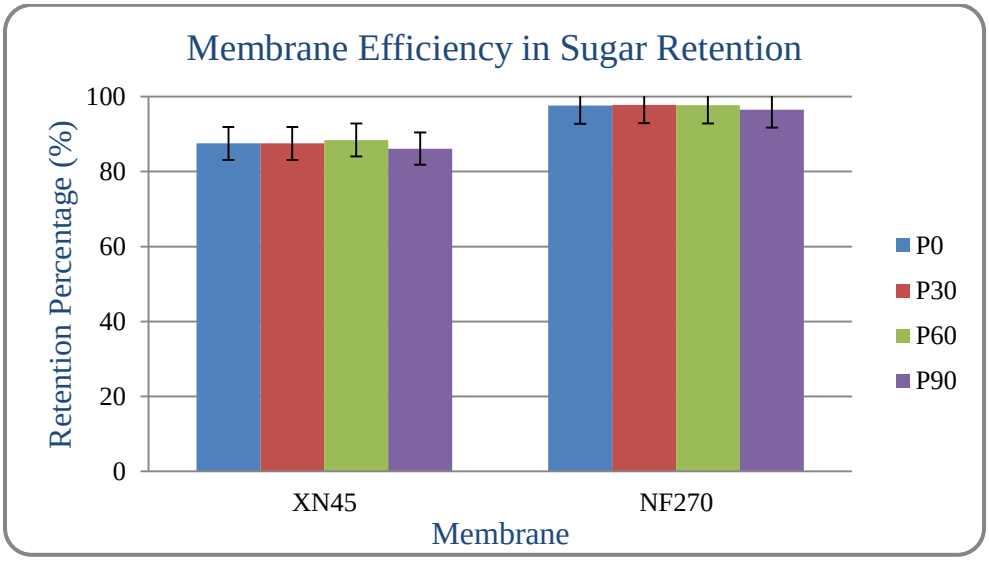

Figure 5-4 : Total sugars retention in hydrolysate by tested membranes at different intervals; first scenario at pH 6 (error bars reflect the percentage errors).

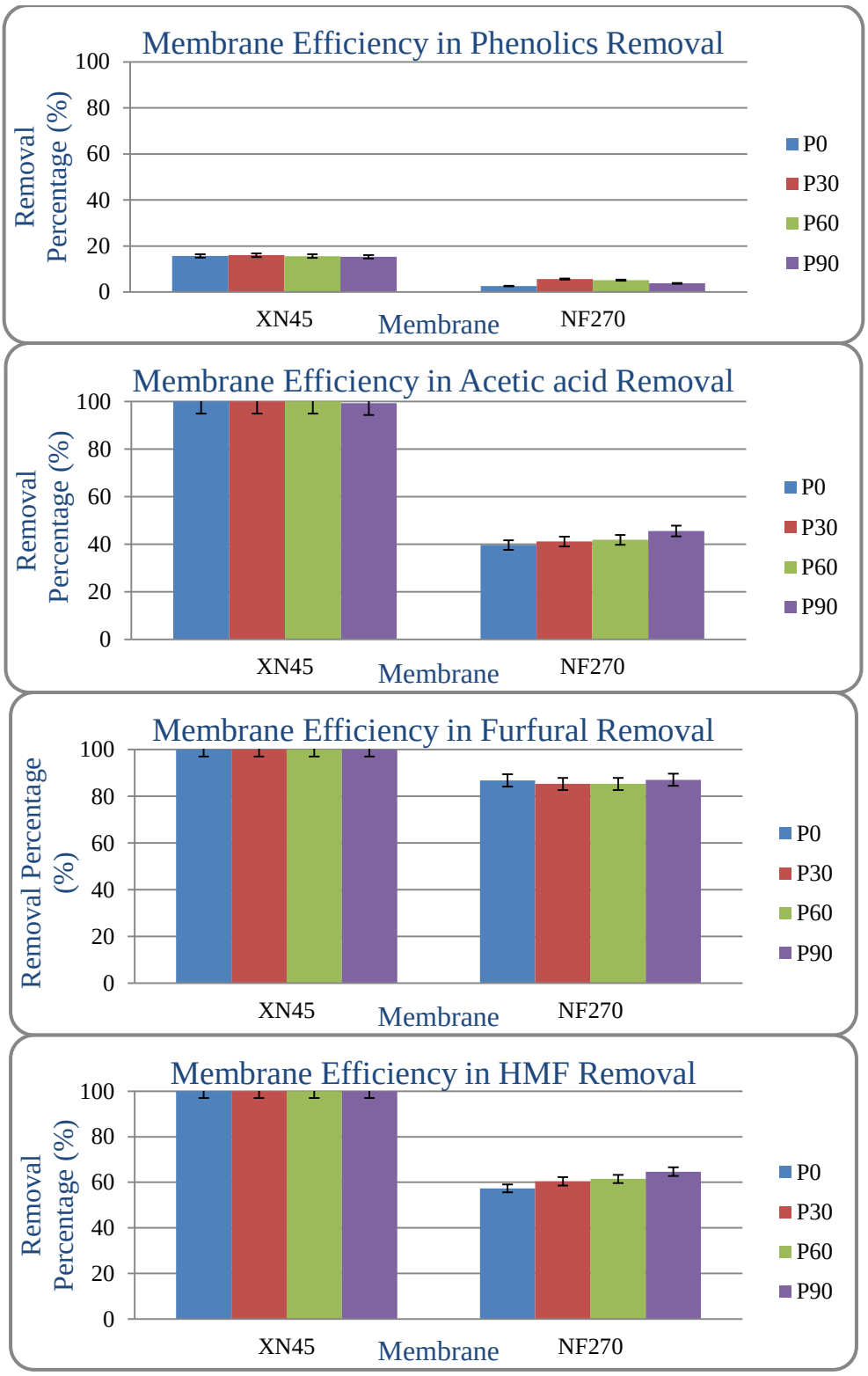

Figure 5-5 : Removal of inhibitors from hydrolysate
by means of membranes at different intervals; first scenario at pH 6
(error bars reflect the percentage errors).

In the case of XN45, this nano-membrane exhibited better performance in terms of inhibitor removals at pH of 6. To describe these behavior, it should be considered that the membrane rejection rates depend on the membrane physico-chemical properties (e.g. hydrophibicity and membrane pore sizes), and solute specifications (e.g. diffusion coefficient and pKa). It is evident that the hydrophilicity and hydrophobicity characteristics affect the fouling and flux of membranes. In fact, with increasing the pH level in the feed solution, the hydrophilic membranes (e.g. XN45) became more open and their flux changed more than hydrophobic membranes. This behavior can be described by the chemical nature of the polymers in the membrane skin layer such as dissociable groups, which make the membrane surface more open and hydrophilic at elevated pH levels.

The XN45 and NF270 membrane surfaces are both made from polyamide, but the differences in their polymerization process or their constituent monomers are due to their surface chemistries.

From these experiments, it can be observed that in terms of inhibitors removal and sugars retention, the XN45 had a better performance than NF270.

In these experiments, in terms of retaining more sugar and removing more inhibitors, the nano-membrane XN45 exhibited better performance and was selected as a suitable membrane in the subsequent steps. Moreover, in terms of sugar retention, the performance of XN45 at pH of 3 was more promising (compared to its performance at pH of 6).

5.2.2 Concentration and dia-filtration

The main goal of dia-filtration was to increase inhibitors removal and purify the retained sugars by dilution of NF (Nano-Filtration) concentrate with deionized water. In this step, the concentration of the concentrate (retentate) was increased by taking out the permeate stream, while the concentrate was recycled back to the feed tank, until a certain volume of concentrate solution was reached. In order to get higher sugars concentration, the concentration step was followed by a dia-filtration step.

5.2.2.1 **Concentration**

The concentration involves the removal of a portion of solution with smaller molecules size than membrane pore size, while keeping the bigger molecules in the concentrate (retentate). With

permeate (filtrate) removal, the fluid concentration increases proportionately. As an example, the solution concentration doubles if the volume decreases by a factor of 2.

In the first scenario, in the concentration step, the volume of 1700 ml of acid hydrolysate was subjected to filtration, which was performed by XN45 (selected membrane from previous step), with a concentration factor of 2 (850 ml concentrate and 850 ml permeate). The permeate solution was continuously removed and collected in a separate vessel until 850 ml of the collected permeate was reached. The time to collect 850 ml of permeate was around 6 hours. A volume of 250 ml of concentrate was kept for further steps (analysis and fermentation). Furthermore, in order to evaluate the performance of the detoxification process, the concentration of inhibitors and sugars was determined in the feed, permeate, and concentrate samples.

5.2.2.2 **Dia-filtration**

To optimize the sugar concentration, two dia-filtration steps were performed:

- First Dia-filtration (1V)

600 ml of concentrated hydrolysate from the last step was diluted with the same amount of deionised water (600 ml). The diluted solution was passed through the membrane (XN45) until the volume of the collected concentrate and permeate was 600 ml. Then a volume of 200 ml of the concentrated stream was separated, and kept for fermentation. The concentrate and permeate samples were analyzed in terms of sugar and inhibitor contents.

- Second Dia-filtration(2V)

400 ml of deionised water were added to 400 ml of concentrated stream from first Dia-filtration. As before, XN45 filtration was performed with a concentration factor of 2 (400 ml of permeate and 400 ml of concentrate) and the collected permeate and concentrate samples were analyzed.

The experimental setup was used in this part was shown in Figure 4-3. The applied mode of separation allowed us to calculate the mass balance (by using the amounts of volumes and concentrations in our solutions). The results are presented in Table 5-4.

Table 5–4[6] : The results of concentration and dia-filtration of hydrolysate (Scenario 1)

Concentration(mg/ L)	Phenols	Acetic acid	Furfural	HMF	Total Sugar
Ai	1783	5160	538	51	44090
Af av dia (conc)	3100	4059	161	26	83140
Af dia 600	2979	2667	74	20	83140
Af dia 400	2672	1752	44	15	73700
Pc av dia	204	4580	729	44	1390
Pc dia 600	147	1310	72	13	840
Pc dia 400	94	2670	167	23	440
Mass Balance (mg)					
Initial Feed	3031	8772	915	87	74953
Concentrate	2635	3450	137	22	70669
Permeate	173	3893	620	37	1182
Concentrate + Permeate	2808	7343	757	60	71851

Whereas:

Ai: Initial Feed (untreated hydrolysate)

Af av dia (conc): Concentrate side from concentration step: first step

Af dia 600: Concentrate side from first dia-filtration (Dia 1V)

Af dia 400: Concentrate side from Second dia-filtration (Dia 2V)

Pc av dia: Permeate side from concentration step

Pc dia 600: Permeate side from first dia-filtration (Dia 1V)

Pc dia 400: Permeate (filtrate) side from second dia-filtration (Dia 2V)

After each step of dia-filtration, a reduction of inhibitors contents in the concentrate stream was observed. However, the results demonstrated that the applied membrane was not effective in terms of phenolics removal.

[6] Note: The mass balance was calculated for concentration step. It indicates that in mass balance calculation, Af av dia (0.85 L) and Pc av dia (0.85 L) samples were considered as concentrate and permeate streams respectively. The volume of the initial feed was 1.7 L.

To investigate the effects of the concentration-detoxification steps on the growth of the solvent-producing microorganism (*C.acetobutylicum ATCC 824*), the samples of Ai (untreated hydrolysate) and the concentrate side of each filtration step (Af, Af dia 600 and Af dia 400), were subjected to fermentation.

5.2.3 Detoxification : concentration before hydrolysis (scenario 2)

The methodology of second scenario is presented in Figure 5-6.

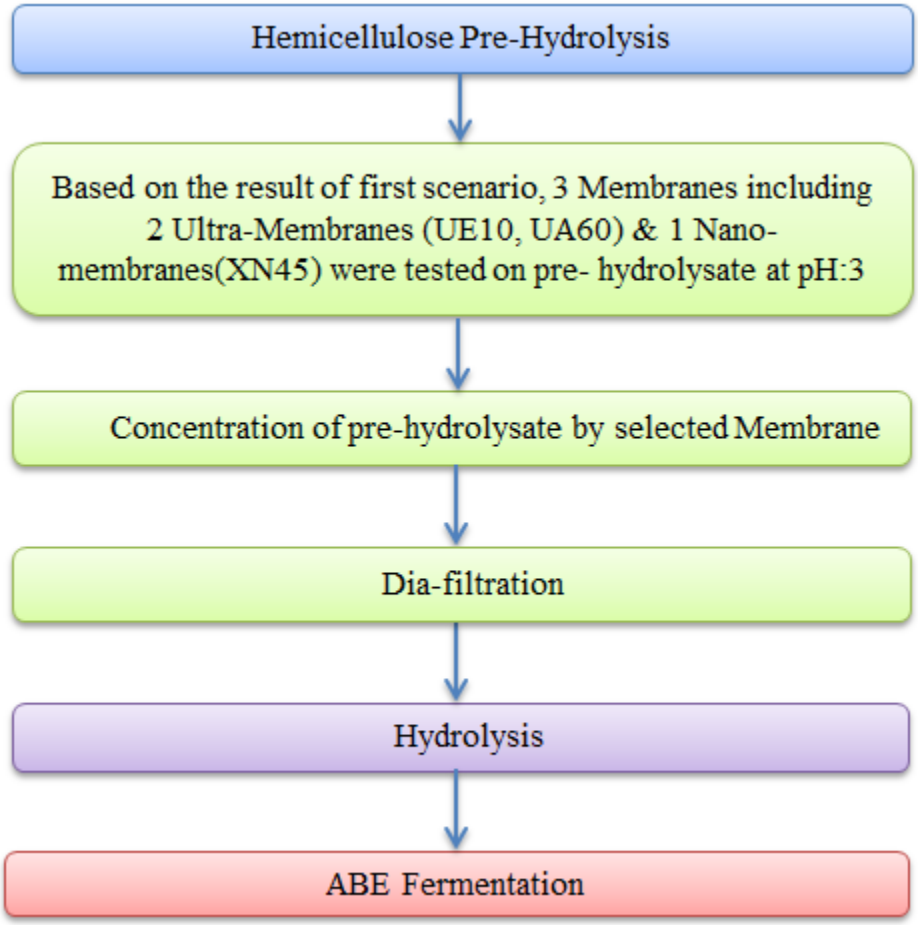

Figure 5-6 : The applied methodology in second scenario (concentration before hydrolysis)

5.2.3.1 **Membrane Selection**

The second scenario in our study was performing concentration process before hydrolysis. For these series of experiments, the procedure was similar to the experiments in the first scenario. However, the previous results (membrane selection in the first scenario) implied that the

membranes XN45, UE10 and UA60 had better performance in terms of inhibitors removal. Related to sugars loss, the performance of the membranes at pH 3 was better than at pH 6. Therefore, in the second scenario, the membranes XN45, UE10 and UA60 were selected and their efficiency in inhibitors removal and sugars retention at pH of 3 was studied. The results are summarized in Figures 5-7 and 5-8 (the related tables are presented in appendix B).

These results indicated that in terms of acetic acid removal, the tested membranes were 100% effective. In terms of elimination of phenolics, furfural and HMF, the membranes UE10 and UA60 showed better results than XN45. However, in terms of retaining sugars, they are not as efficient as XN45, which offered the least sugar losses among the applied membranes. Similar to the experiments in the first scenario, the high inhibitors removal and low sugars retention of UE10 and UA60 are due to their high molecular weight cut-off, which are higher than the molecular weight of the targeted compounds (phenolics, acetic acid, furfurals, HMF and sugars).

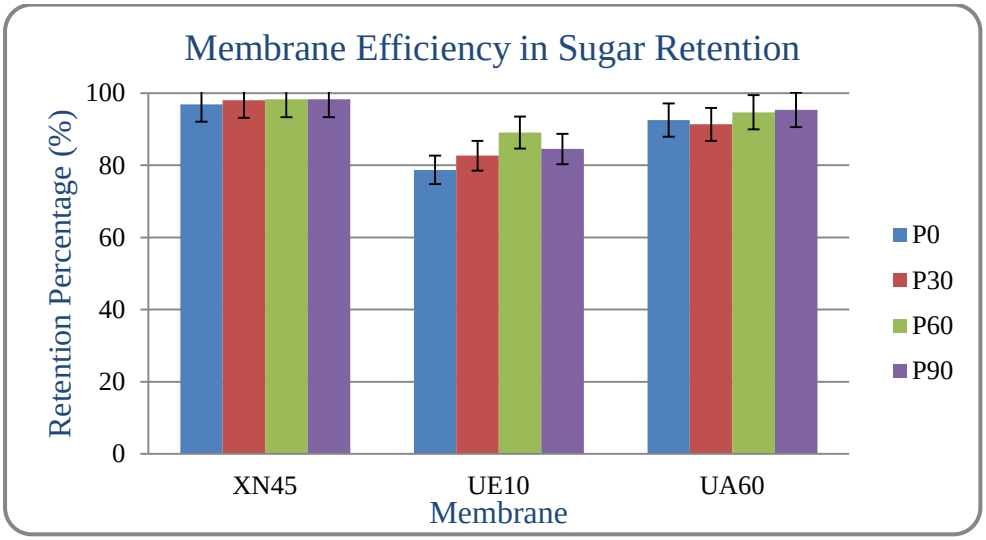

Figure 5-7 : Total sugars retention in hydrolysate by tested membranes at different intervals; second scenario at pH 3 (error bars reflect the percentage errors).

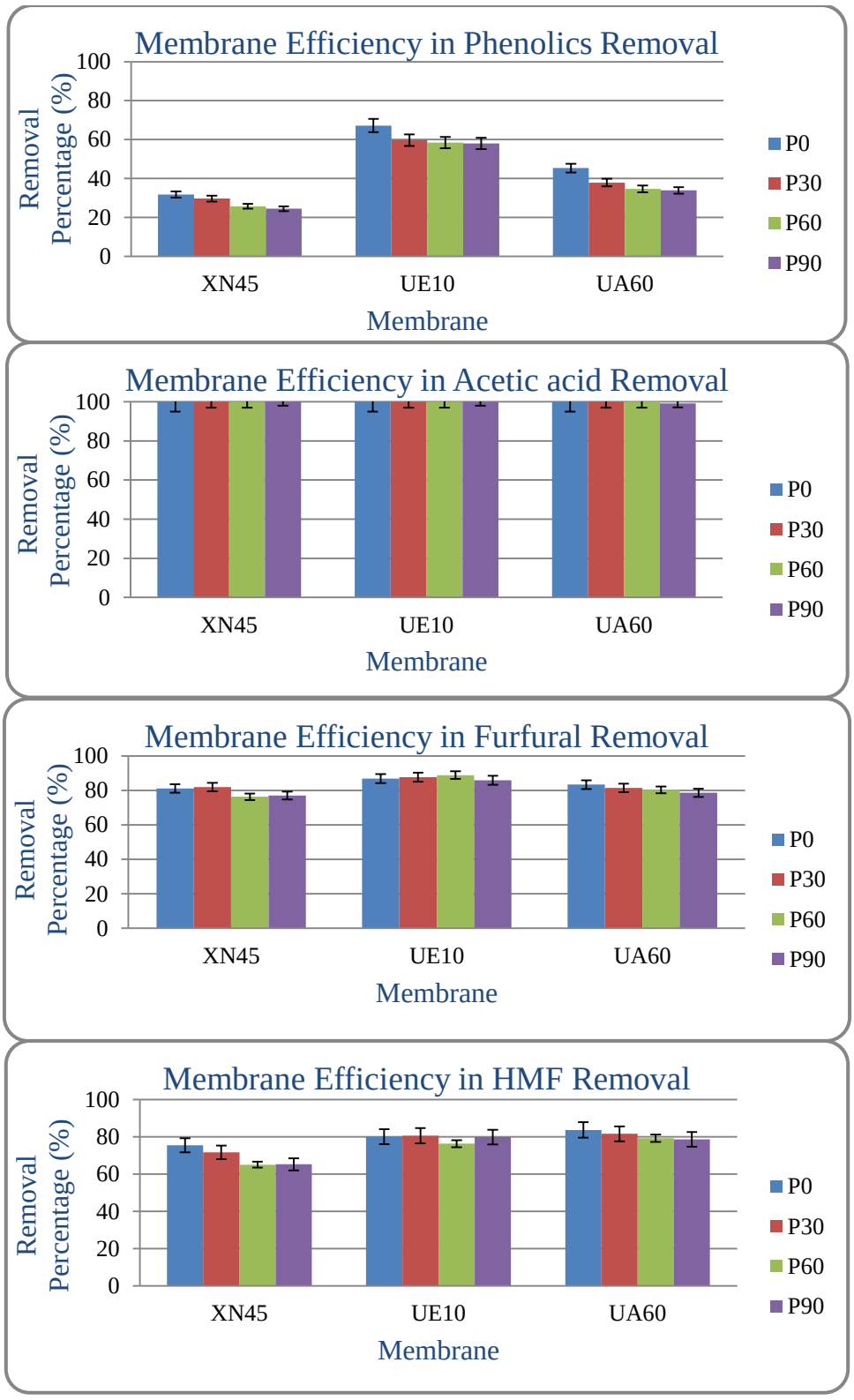

Figure 5-8 : Removal of inhibitors from hydrolysate
by means of membranes at different intervals; second scenario at pH 3
(error bars reflect the percentage errors).

According to the results, in the second scenario, the efficiency of tested membranes in terms of furans removal decreased,while their efficency in sugar retention and phenolics removal was higher than in the first scenario at corresponding pH (=3). This behavior could be described by the effect of solute concentration. Aydoğan suggested that in the multi-component solution, the membrane performance might be affected by interactions between solutes. Previous studies in the literature have demonstrated that the simultaneous increase of the furfural and xylose concentrations resulted in the reduction of the furfural rejection by nano-membranes (Aydoğan et al., 1998; Qi et al., 2012).

Finally based on the generated results in both scenarios and since the XN45 at pH 3 showed better performance in terms of inhibitors removal and sugars rejection, XN45 was selected as a suitable membrane for further steps. To get higher sugars concentration and increase the inhibitors removal, the dia-filtration process was performed as a supplementary step.

Moreover, to detect the fouling effects on the XN45, the pure water permeability, before and after filtration, was measured (Equation 3). The difference in the pure water permeability (PWP) indicates the fouling phenomenon in the filtration process. This behavior is inherent in the nature of solutions and existence of organic matter contents, which are mostly hydrophobic. Hence, they can be rejected by membrane and accumulate on the membrane surface, which result in permeate flux reduction. These fouling effects can be limited by: membrane cleaning and backwashing, optimization of the operation conditions, applying cross flow filtration with high velocity, feed pre-treatment in order to limit its fouling tendency (e.g. controlling organic matters), and modifying the antifouling characteristics of membrane (e.g. increasing the hydrophilicity of the membrane surface)(Abdelrasoul et al., 2013).

5.2.3.2 **Concentration / Dia-filtration /Hydrolysis**

In the second scenario, first a volume of 2L of pre-hydrolysate was subjected to filtration in a concentration mode by using XN45. After collection of a certain volume of concentrate solution (1L), in the next step, the obtained concentrate stream was diluted with the same volume of deionized water (1 L), and then passed through XN45 (Dia-filtration 1V) until the volume of collected concentrate and permeate was 1000 ml. Then the concentrate side was subjected to hydrolysis (same to the first scenario), followed by filtration with microfiltration membrane

(pore size 1.5 µm). After performing acid hydrolysis, the sugars content and inhibitors concentration in the hydrolysate solution were analyzed. Afterwards, the samples of concentrate side of dia-filtration step (Af dia 1L) and untreated pre-hydrolysate (Ai) were subjected to the fermentation. The results are shown in Table 5-5.

Table 5–5[7] : The concentration and dia-filtration of pre-Hydrolysate (Scenario 2)

Concentration(mg/L)	Phenols	Acetic acid	Furfural	HMF	Total Sugar
Ai	560	2455	583	41	20110
Af av dia	760	2591	519	52	44930
Af dia 1L	537	1689	216	35	43400
Pc av dia	N/A	2308	495	29	490
Pc dia 1L	N/A	1374	243	16	190
Af dia 1L after Hydrolysis	3031	7773	902	84	68830
Mass Balance (mg)					
Initial Feed	1120	4909	1166	82	40220
Concentrate	760	2591	519	52	44930
Permeate	N/A	2308	495	29	490
Concentrate + Permeate	N/A	4899	1014	80	45420

Whereas:

Ai: Initial Feed: Untreated Pre-hydrolysate (volume: 2L)

Af av dia: Concentrate side from concentration step: first step (volume: 1L)

Af dia 1L: Concentrate side from first dia-filtration (Dia 1V)

Pc av dia: Permeate side from concentration step (volume: 1L)

Pc dia 1L: Permeate side from first dia-filtration (Dia 1V)

As it can be seen in Table 5-5, similar to the first scenario, by applying a concentration step, a significant increase in sugars concentration and decrease in inhibitors contents were observed.

[7] Note: Same as first scenario, the mass balance was calculated for concentration step. It indicates that in mass balance calculation, Af av dia (1 L) and Pc av dia (1 L) samples were considered as concentrate and permeate streams respectively. Also the volume of initial feed was 2 L.

Essentially, with the detoxification step in both scenarios, the objective was to obtain higher sugars concentration and to remove more inhibitors. In the first scenario, the sugars concentration (mainly xylose) in the concentrate stream increased from 44.1 g/L to 83.1 g/L, i.e. 88% increase in sugar concentration was achieved. In addition, the low sugar concentration in the permeate solution demonstrates negligible sugar loss. Furthermore, the contents of phenolics, acetic acid, furfural and HMF in the concentrate stream decreased by 13%, 61 %, 85% , and 75% respectively (based on the mass balance calculations). The presence of inhibitors in the concentrate solution can exhibit inhibition on the microbial growth during fermentation, where the concentrate solution is applied as a carbon source. Therefore, in order to remove more inhibitors and get higher sugars concentration, a dia-filtration was performed. In the first scenario, the removal percentage of phenolics, acetic acid, furfural and HMF from concentration step increased from 4%, 34%, 54% and 23% in the first dia-filtration to 14%, 57%, 73% and 42% in the second dia-filtration, respectively (based on inhibitors concentration in concentrate side before and after dia-filtration step). However, the reduction of sugars concentration (decreased from 83 g/L to 73.7 g/L) after the second dia-filtration step, indicates that sugar loss occurred.

In the second scenario, after the concentration step, the concentration of sugars in the concentrate solution (retentate) increased by 123%, and reached from 20 g/L to 44.9 g/L. In terms of inhibitors removal, the contents of phenolics, acetic acid, furfural and HMF in the concentrate (retentate) solution decreased by 32%, 47%, 55% and 37% respectively (based on the mass balance calculations). At the end, by applying the dia-filtration step, the concentration of phenolics, acetic acid, furfural and HMF in concentration step (concentrate stream) decreased by approximate 29% ,35% , 58% and 33%, respectively. However, it should be considered that by performing the hydrolysis step after detoxification in the second scenario, a significant amount of inhibitors were generated and additional filtration would be required.

The comparison between the results obtained from both scenarios has demonstrated that in terms of sugars recovery and final inhibitors contents, the first scenario was more effective than the second scenario. However, as discussed earlier, it should also be noticed that the second scenario has its own advantages (e.g. saving more energy and using lower chemicals during hydrolysis step), and applying an additional filtration step after hydrolysis may improve its effectiveness.

The concentrated-detoxified pre-hydrolysate and hydrolysate samples, in addition to the untreated pre-hydrolysate and hydrolysate, were subjected to fermentation.

5.3 Fermentation

As previously discussed, the last step of the experimental work was fermentation (a common step for both scenarios). The detoxified hydrolysates were used as fermentation feeds. There were six samples in total, four of them were concentrated-detoxified samples (three from scenario 1 and one from scenario 2), and two were non-detoxified samples (pre-hydrolysate and hydrolysate). In addition, four control samples (two positive controls and two negative controls) were used. The contents of the fermentation feeds, in terms of inhibitors and sugars concentration, are given in Table A-11 (Appendix C).

The *Clostridium acetobutylicum ATCC824*, the most commonly applied strain for ABE fermentation (Ramey et al., 2004), was considered as a solvent-producing microorganism.

The fermentation experiments were carried out in a 250-ml Erlenmeyer flask with 150 ml working volume for 100 h.

5.3.1 Media and growth conditions

In our study, as indicated before, two cultural mediums were employed for cultivation of *C. acetobutylicum ATCC824*:

- Reinforced Clostridial Medium(RCM)
- Synthetic Medium (SM)

The mediums for fermentation experiments were made anaerobic by 3-5 minutes sparging of nitrogen-carbon dioxide gas mixture (Van Andel et al., 1985) in the ratio of 80:20. Erlenmeyer flasks (250 ml) containing 150 ml of detoxified samples were inoculated with 2% (vol /vol) of highly motile cells of pre-cultured *C. acetobutylicum ATCC824*. The fermentation experiments were performed anaerobically in an incubator / shaker (New Brunswick Scientific) set at 70 RPM and 35 °C.

In addition, anaerobic fermentation using 60 g/L xylose in RCM (Reinforced Clostridial Medium) and synthetic medium were used as positive controls, while fermentation of two mentioned medium containing furfural (350 mg/L), HMF (50 mg/L), acetic acid (12 g/L) and gallic acid (4 g/L) were used as the negative controls.

During fermentation experiments, the microbial growth in the samples was investigated. However, factors such as microbial contaminants and/or oxygen presence can result in unusual behavior of *C. acetobutylicum* cells and may influence the growth characteristics of the microorganism during fermentation.

The bacterial growth profile of the control samples in both employed mediums is shown in Figure 5-9.

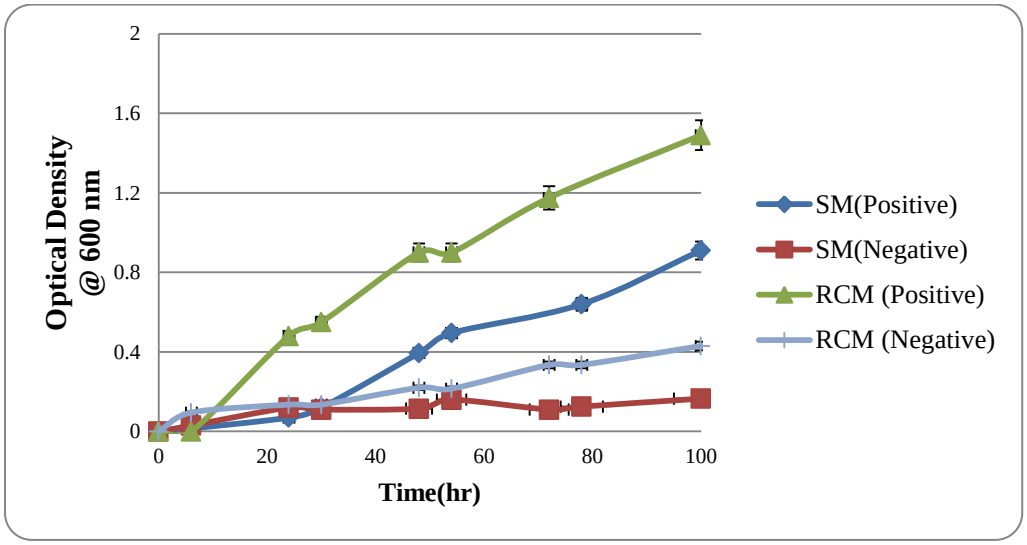

Figure 5-9 : The growth of microorganism in control samples
(error bars reflect the percentage errors based on the analysis replication).

Based on the data of Figure 5-9, the maximum optical density (cell concentration) was around 1.5 and was observed in RCM-positive, while the highest optical density in SM-positive was around 0.9. This indicates that the microorganism had better growth in the medium of RCM-positive control. In negative controls, the observed cell density in RCM was also higher than SM, which demonstrates that RCM medium could provide a suitable biophysical and biochemical (nutritional) environment for *C. acetobutylicum ATCC824*, and can be considered as a better culture medium in comparison with SM. The biomass formation in the samples generated in the previous steps was also studied. The time-courses of cell growth in the fermentation samples are illustrated in Figure 5-10.

As can be seen in Figure 5-10, among the fermented samples, the maximum length of lag phase and the minimum biomass formation was observed in the non-detoxified pre-hydrolysate. This is

due to the low concentration of sugar monomers in this sample. Thus, the lower fermentable sugars would limit the biomass formation and prolong the lag phase.

Moreover, due to the high inhibitors concentration in the negative controls, the microorganism cells were not able to grow properly in these mediums.

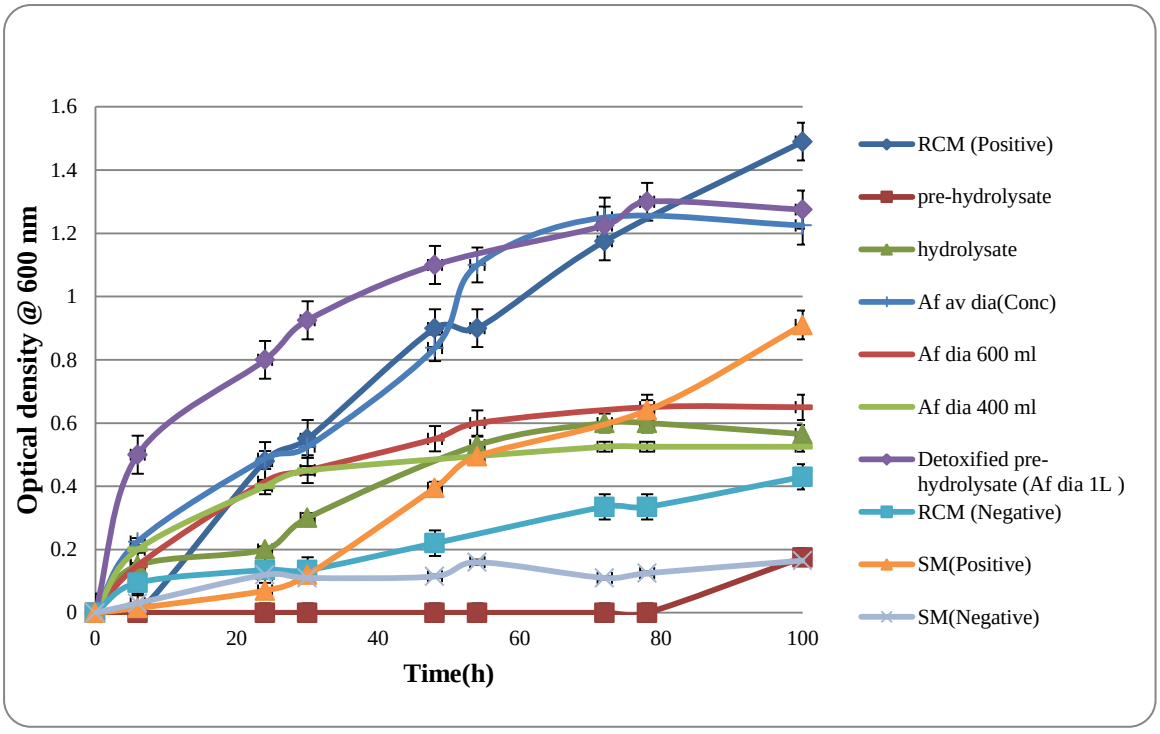

Figure 5-10 : Cell density at different times in fermented samples
(error bars reflect the percentage error based on the analysis replication).

In detoxified samples, the maximum cell concentrations have been observed in the sample of detoxified pre-hydrolysate (Af dia 1L) from the second scenario with an OD_{600} of 1.3 at 78h, and detoxified hydrolysate (Af av dia–conc) from the first scenario with an OD_{600} of 1.2 at 72h. These amounts of cell density were slightly higher than the result of RCM positive that was 1.18 at 72 h. These observations confirmed that in terms of cell growth profile, the lower inhibitor contents could lead to observe higher cell density, while the higher initial sugars concentration could result in faster cell growth. Furthermore, in the positive controls, despite zero concentration of inhibitors, the maximum cell density in detoxified samples was achieved in shorter time, and the exponential growth phase was shortened. However, the biomass formation and exponential

cell growth phase could continue in positive controls, and it can be due to the lack of inhibitors in these samples. In other words, the existence of inhibitors can limit the biomass production.

The comparison of two detoxified samples of Af av dia (conc) and Af dia 600 from the first scenario, demonstrated that despite having similar concentration of phenolics and sugars, the higher cell density was observed in the sample with higher contents of acetic acid and furans. These conclusions indicate acetic acid and furans may have positive effect on cell growth and biomass formation, rather than to be toxic. A similar effect has also been observed for *C.beijerinckii BA101* by Cho and his colleagues (Cho et al., 2009). Furthermore, it has been reported that by addition of < 10 g/L of acetic acid, the slight increasing of ABE concentration by solventogenic clostridia was observed; while in the presence of 11.7 g/L acetic acid this amount reduced drastically (Cho et al., 2012).

In the first scenario, the cell concentration in Af av dia (conc) was higher than Af dia 600 and Af dia 400, and by decreasing the acid acetic, the reduction of maximum cell density was observed. These results can be due to the existence of microbial contaminants, which use acetic acid as a carbon source (acetobacter and / or acidomonas). Therefore, the samples were analysed under an optical microscope and the presence of at least two other types of microorganisms in addition to *Clostridium* bacteria was confirmed. In fact, due to the restriction associated with *Clostridium* strains, different contaminants (e.g. presence of oxygen) can influence the activity and growth behavior of microorganisms. To validate the detoxification effectiveness for butanol production and to investigate the effects of inhibitors on microbial growth profile and solvent production, all the microbial manipulations (e.g. sampling, monitoring, etc.) must be performed sterilely.

It should also be noted that the toxic effects of acetic acid (pKa = 4.75 at 25°C) are mainly pH dependent. Where the pH level of the solution is lower than its pKa, the dominant form of acetic acid is the protonated form. This form of acetic acid is lipophilic, which is able to pass through the cytoplasmatic membrane and exhibit negative effects on the cell metabolism (Han et al., 2006). Based on the literature review, high concentrations of acetic acid and butyric acid can have toxic effect on the cell growth (Costa et al., 1983) and the lower pH can result in the lower cell biomass.

In the case of phenolic compounds, typically the lignin derived phenolic compounds show the hydrophobic behaviors. In general, the hydrophobicity can be considered as toxicity indicator of

organic compounds, and in most studies, the phenolic compound due to their high hydrophobicity effects, are more toxic than furans and weak acids.

The data in the negative controls indicated that phenolic compounds, and acetic acid (>10g/L) can inhibit the biomass formation. However, under certain concentrations, high initial sugar contents and stimulatory effects of furan compounds on the cell concentration can limit their inhibitory effects.

SUMMARY, CONCLUSION AND RECOMMENDATIONS

• Summary and conclusion

This research was conducted to investigate the impacts of membrane separation on partial or complete removal of potential inhibitors from hemicellulosic hydrolysate. In this study, the membrane separation experiments were performed in two main phases: membrane selection and detoxification. In the proposed methodology, two detoxification scenarios, including concentration after hydrolysis and concentration before hydrolysis were conducted and their performances were compared.

In the first part of the study (membrane selection), in order to find a suitable membrane (able to remove more inhibitors and retain more sugars), hydrolysate and pre-hydrolysate were subjected to ultra-filtration and nano-filtration, which based on the filtration spectrum are effective processes for sugar concentration. Five commercial membranes including nano-membranes and ultra-membranes with different range of molecular weight cut off (M.W.C.O) were used, and their performances in terms of inhibitors removal and sugars retension were compared at pH levels of 3 and 6. The results of this set of experiments allowed us to evaluate the separation performance of the tested membranes and to compare their efficiency under the same operating conditions. The results in both scenarios indicated that in terms of inhibitors elimination and sugars retention, the XN45 (Nano- membrane) was more effective than the other membranes. In addition, due to the interactions between membranes and solute molecules, a better separation was obtained at pH of 3. The experiments proved that in terms of retaining sugars and removing acetic acid, furfural, and HMF, the membrane filtration exhibited more efficient performance than phenolic removal.

Moreover, the results of this part demonstrated that the membrane cut-off had a significant effect on the filtration performance. However, it should be considered that in membrane separation, factors such as membrane physico-chemical characteristics and their nature (e.g. the hydrophobicity and hydrophobilicity effects of membranes, charge of membrane surface), solute properties (e.g. solute diffusion coefficient), organic matter structure, operational conditions, and hydrodynamic parameters (e.g. feed flow rate), can also influence the rejection rate of the membranes. As an example, the polyamide-based membranes (e.g. XN45) present negative

charges on their surfaces and consequently the substances with negative charges would be rejected due to the electrostatic forces. Therefore, substances with a pKa value lower than the applied pH could be dissociated; consequently, their anionic form could be repulsed by negatively charged surface of membrane, which results in a lower concentration of substance in permeate streams. On the contrary, this behavior is not typical for non-ionic substances.

One of the main results arising from the performances of nano-filtration and ultra-filtration in this work was that it could be misleading to characterise the fractions, which were obtained by membrane filtration, only according to the molecular weight cut-off of the membrane. This indicated that even when high volume reduction factor are gained, some other molecular weight classes can be found in different fractions

In the next part of the study, which was the detoxification step, two different scenarios were conducted: concentration after hydrolysis and concentration before hydrolysis, and their performances were compared. In order to get higher sugar concentration and to increase the inhibitor removal, the batch filtration in concentration mode followed by dia-filtration step(s) with the membrane XN45 was suggested. By applying this method a significant enhancement of sugars concentration and inhibitors removal in both scenarios, was obtained. However, the overall results of this study indicated that in terms of phenolics elimination, the performance of membrane separation was not effective. This behaviour can be explained by the high hydrophobilicity characteristics of the phenolic compounds. To complete the removal of these components, a secondary detoxification step such as the use of enzymes or activated charcoal can be applied in combination with membrane separation.

The effectiveness of both suggested scenarios were compared, the results indicated that the first scenario (concentration after hydrolysis), was more promising than the second scenario. By applying the concentration after the hydrolysis step, a solution with higher sugar concentration and lower inhibitors contents can be obtained.

It should also be considered that although dia-filtration process generated purified solution with small sugar loss, it utilized a large volume of water, which can be a limiting factor in industrial applications. Therefore, in order to minimize the water consumption, it is essential to optimize the dia-filtration process and to obtain an optimized dilution volume factor, at which the inhibitor concentration is lower than the concentration representing toxic effects on the microbial

metabolisms. On the other hand, water consumption can also be reduced by modifying the strain and making it resistant to a higher concentration of inhibitors (as much as possible), while still keeping high yield and product concentration.

The last step in our study was fermentation, where the growth profile of *C. acetobutylicum* was studied. The concentrated-detoxified solutions of both scenarios, the untreated hydrolysate and pre-hydrolysate solutions, and the positive and negative controls were used as fermentation feeds.

In particular, the conclusions from the fermentation step are:

- Lower fermentable sugars would limit the biomass formation and prolong the lag phase;
- The higher sugars concentration can result in faster cell growth and shorten the growth of the exponential phase;
- The presence of the inhibitors can limit the biomass production;
- The extending of exponential growth phase and cell biomass production can be achieved by decreasing the inhibitor contents (e.g. positive controls);
- Furfural and HMF contents can have stimulatory effects on the cell growth and biomass formation rather than to be toxic;
- Acetic acid under certain concentrations may exhibit positive effects on biomass production rather than inhibitory effects;
- The excess production of organic acids in the medium can have negative effects on the ABE fermentation;
- In addition to the presence of the inhibitors, there is also the possibility of the existence of other unknown substances (e.g. potentially toxic metallic ions including Fe and Cr) in hydrolysate, which may have an inhibitory effect on the cell metabolisms.

To achieve high fermentation efficiency, it should be considered that:

- The pH profile during fermentation should be controlled rather than let it self-controlled or keep it as a constant;
- The optimum conditions for each bacteria strain and substrate are case specific and should be investigated separately;
- The analysis of acid production (mainly butyric acid and acetic acid) during fermentation can give a better knowledge of the phenomena occurring in ABE fermentation.

• Recommendations

The following items are recommended for future research:

- To evaluate the effects of initial sugars concentration on the ABE fermentation in order to determine the optimal sugars concentration for butanol production;
- To evaluate the effects of inhibitors (e.g. phenolics, acetic acid and furans) on the ABE production of *C. acetobutylicum;*
- To investigate the cell tolerance to different inhibitors and final product (butanol);
- To combine another detoxification method with membrane separation to complete removal of phenolics;
- Applying another filtration step in the second scenario (after hydrolysis step) in order to make it more efficient;
- Evaluation of synergisms between different inhibitors on both microbial growth rate and fermentation process, and documenting the interactions between different toxic materials on sugar conversion;
- To investigate the effects of biomass formation on the ABE production.

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APPENDIX A - MEMBRANE SELECTION (Scenario 1)

(Concentration after hydrolysis)

Table A-1 : The result of filtration of hydrolysaye by NF90 at pH 3

	[Phenolics] g/L	[Acetic acid] g/L	[Furfural] g/L	[HMF] g/L	[Total Sugar] g/L
Ai	1.416	5.37	0.57	0.05	28.18
P0	0.014	1.02	0.11	0.00	0.22
P30	0.013	1.01	0.12	0.00	0.20
P60	0.010	0.83	0.10	0.00	0.12
P90	0.009	0.74	0.09	0.00	0.11
Af		5.07	0.50	0.04	28.86
Removal percentage (%)					
P0	0.96	18.95	18.48	5.38	0.79
P30	0.94	18.75	21.77	3.37	0.73
P60	0.72	15.43	17.95	2.46	0.43
P90	0.63	13.87	15.65	2.39	0.38

Table A-2 : The results of filtration of hydrolysaye by XN45 at pH 3

	[Phenolics] g/L	[Acetic acid] g/L	[Furfural] g/L	[HMF] g/L	[Total Sugar] g/L
Ai	1.27	5.09	0.50	0.04	30.08
P0	0.20	3.85	0.40	0.03	1.68
P30	0.20	3.83	0.47	0.04	1.07
P60	0.17	3.69	0.45	0.03	1.23
P90	0.14	3.46	0.43	0.03	1.07
Af		4.61	0.40	0.04	28.94
Removal Percentage (%)					
P0	16.09	75.67	79.61	80.07	5.59
P30	15.67	75.26	93.29	87.61	3.57
P60	13.14	72.58	89.86	76.39	4.07
P90	11.02	68.04	85.89	70.21	3.57

Table A-3 : The results of filtration of hydrolysaye by UE10 at pH 3

	[Phenolics] g/L	[Acetic acid] g/L	[Furfural] g/L	[HMF] g/L	[Total Sugar] g/L
Ai	1.15	3.66	0.39	0.04	22.24
P0	0.49	4.69	0.38	0.04	13.64
P30	0.47	4.73	0.38	0.04	12.11
P60	0.45	4.68	0.38	0.04	12.19
P90	0.44	4.70	0.37	0.04	12.04
Af		4.48	0.37	0.03	20.33
Removal percentage (%)					
P0	42.91	100.00	99.36	96.04	61.31
P30	41.29	100.00	99.26	95.20	54.47
P60	39.10	100.00	98.76	93.78	54.81
P90	38.34	100.00	96.42	97.99	54.13

Table A-4 : The results of filtration of hydrolysaye by NF270 at pH 3

	[Phenolics] g/L	[Acetic acid] g/L	[Furfural] g/L	[HMF] g/L	[Total Sugar] g/L
Ai	1.31	3.51	0.33	0.03	19.95
P0	0.06	2.54	0.26	0.02	0.56
P30	0.07	2.47	0.26	0.02	0.51
P60	0.06	2.28	0.24	0.01	0.46
P90	0.05	2.27	0.24	0.01	0.44
Af		3.26	0.25	0.03	21.32
Removal percentage (%)					
P0	4.82	72.24	79.53	50.00	2.81
P30	5.11	70.15	79.28	50.84	2.57
P60	4.83	64.78	73.14	44.27	2.31
P90	4.00	64.48	72.09	44.37	2.23

Table A-5 : The results of filtration of hydrolysaye by UA60 at pH 3

	[Phenolics] g/L	[Acetic acid] g/L	[Furfural] g/L	[HMF] g/L	[Total Sugar] g/L
Ai	1.48	4.15	0.57	0.05	42.64
P0	0.28	4.00	0.57	0.05	5.34
P30	0.26	3.93	0.59	0.05	5.37
P60	0.27	4.03	0.58	0.05	4.85
P90	0.30	3.67	0.57	0.05	4.52
Af		3.72	0.47	0.04	50.18
Removal percentage (%)					
P0	18.95	96.21	99.88	100.00	12.51
P30	17.77	94.70	100.00	100.00	12.59
P60	18.13	96.97	100.00	100.00	11.36
P90	19.99	88.38	100.00	100.00	10.61

Table A-6 : The results of filtration of hydrolysaye by XN45 at pH 6

	[Phenolics] g/L	[Acetic acid] g/L	[Furfural] g/L	[HMF] g/L	[Total Sugar] g/L
Ai	1.11	1.54	0.22	0.02	16.53
P0	0.17	1.55	0.22	0.03	2.06
P30	0.18	1.55	0.24	0.03	2.06
P60	0.17	1.56	0.23	0.03	1.91
P90	0.17	1.53	0.22	0.03	2.29
Af		1.48	0.20	0.02	17.37
Removal percentage (%)					
P0	15.68	100.00	100.00	100.00	12.48
P30	15.99	100.00	100.00	100.00	12.48
P60	15.58	100.00	100.00	100.00	11.56
P90	15.27	99.32	100.00	100.00	13.86

Table A-7 : The results of filtration of hydrolysaye by NF270 at pH 6

	[Phenolics] g/L	[Acetic acid] g/L	[Furfural] g/L	[HMF] g/L	[Total Sugar] g/L
Ai	0.98	1.43	0.18	0.02	20.11
P0	0.02	0.57	0.15	0.01	0.47
P30	0.06	0.59	0.15	0.01	0.44
P60	0.05	0.60	0.15	0.01	0.45
P90	0.04	0.65	0.15	0.01	0.69
Af		1.32	0.17	0.02	24.07
Removal percentage (%)					
P0	2.54	39.71	86.80	57.37	2.35
P30	5.65	41.18	85.30	60.44	2.21
P60	5.19	41.91	85.31	61.48	2.26
P90	3.79	45.59	87.07	64.68	3.45

APPENDIX B –MEMBRANE SELECTION (Scenario 2)

(Concentration before hydrolysis)

Table A-8 : The results of filtration of pre-hydrolysaye by XN45 at pH 3

	[Phenolics] g/L	[Acetic acid] g/L	[Furfural] g/L	[HMF] g/L	[Reducing Sugar] g/L
Ai	0.56	1.72	0.68	0.05	44.32
P0	0.18	2.02	0.55	0.04	1.38
P30	0.17	1.91	0.56	0.04	0.86
P60	0.15	1.84	0.52	0.03	0.77
P90	0.14	1.83	0.52	0.03	0.77
Af		1.78	0.63	0.05	15.23
Removal percentage in permeate (%)					
P0	31.83	100.00	81.05	75.47	3.11
P30	29.69	100.00	81.95	71.67	1.94
P60	25.75	100.00	76.27	65.08	1.73
P90	24.50	100.00	76.98	65.25	1.75

Table A-9 : The results of filtration of pre-hydrolysaye by UE10 at pH 3

	[Phenolics] g/L	[Acetic acid] g/L	[Furfural] g/L	[HMF] g/L	[Reducing Sugar] g/L
Ai	0.56	1.52	0.51	0.04	24.07
p0	0.37	1.63	0.44	0.03	5.12
P30	0.33	1.67	0.45	0.03	4.18
P60	0.33	1.65	0.45	0.03	2.63
P90	0.32	1.67	0.44	0.03	3.73
Af		1.49	0.49	0.04	38.84
Removal percentage in permeate (%)					
p0	67.21	100.00	86.83	80.11	21.29
P30	59.72	100.00	87.62	80.64	17.36
P60	58.48	100.00	88.81	76.34	10.92
P90	58.02	100.00	85.80	79.87	15.49

Table A-10 : The results of filtration of pre-hydrolysaye by UA60 at pH 3

	[Phenolics] g/L	[Acetic acid] g/L	[Furfural] g/L	[HMF] g/L	[Reducing Sugar] g/L
Ai	0.54	1.30	0.44	0.04	29.24
P0	0.25	1.34	0.37	0.03	2.19
P30	0.21	1.34	0.36	0.03	2.53
P60	0.19	1.31	0.35	0.03	1.56
P90	0.18	1.29	0.34	0.03	1.36
Removal percentage in permeate (%)					
P0	45.38	100.00	83.30	83.68	7.49
P30	37.93	100.00	81.39	81.62	8.64
P60	34.71	100.00	80.26	79.27	5.33
P90	33.89	99.19	78.57	78.69	4.66

APPENDIX C - The contents of Fermentation Feeds

Table A-11 : The contents of fermentation feeds

Concentration (mg/L)		Phenolics	Acetic acid	Furfural	HMF	Sugar
Non-detoxified samples	Pre-hydrolysate	560	2455	528	40	20100
	Hydrolysate	1783	5160	538	51	44090
Detoxified Hydrolysate (Scenario 1)	Af av dia(Conc)	3100	4059	161	26	83140
	Af dia 600	2979	2667	74	20	83140
	Af dia 400	2672	1752	44	15	73700
Detoxified Pre-hydrolysate (Scenario2)	Af dia 1L (After filtration)	567	1689	216	35	43400
Control	RCM (Negative)	4000	12000	350	50	60000
	RCM (Positive)	0	0	0	0	60000
	SM (Negative)	4000	12000	350	50	60000
	SM (Positive)	0	0	0	0	60000